PLANT PHYSIOLOGY

VOLUME 35 JULY, 1960 NUMBER 4

# MOVEMENT AND DISTRIBUTION OF RADIOPHOSPHORUS IN CROWNS OF HEALTHY AND POLE BLIGHTED WESTERN WHITE PINES (PINUS MONTICOLA D. DON.) [1, 2]

WILLIAM K. FERRELL[3], FREDERIC D. JOHNSON, AND CHRISTIAN E. MICHELSEN[4]

FOREST WILDLIFE, AND RANGE EXPERIMENT STATION, COLLEGE OF FORESTRY, UNIVERSITY OF IDAHO, MOSCOW

## INTRODUCTION

Pole blight is a disease or abnormal condition of western white pine affecting trees in northern Idaho, western Montana, eastern Washington, and southern British Columbia. It is restricted to pole-sized trees and is characterized by a yellowing of the foliage of the upper crown of the tree and at times by cambial necrosis usually accompanied by resinosis of the stem, general growth reduction, and always followed by decline and death of the tree. The status of the disease has been reported by Wellner (8) and by Wright and Graham (9), and the symptoms have been described in detail by Buchanan, et al (3). In spite of the considerable amount of investigation on the disease, its cause is not yet known.

Analyses in our laboratory have shown that phosphorus is somewhat higher in concentration in the yellow foliage in the upper crown of the diseased trees as compared with the concentration in the green foliage of adjacent healthy trees. In contrast, most other elements are found in lower concentration in the foliage in the upper crown of the diseased trees as compared with that of the healthy ones. For this reason, it was decided to study the differences in translocation and deposition of phosphorus in these trees with the use of phosphorus 32.

## MATERIALS AND METHODS

Areas in the Deception Creek Experimental Forest within the Coeur d'Alene National Forest and an area near Clarkia in the St. Joe National Forest were selected for the study. Some trees in the work areas had typical pole blight symptoms, were 60 to 70 years old, and were 65 to 90 ft in height. The experimental work was carried out during the summers of 1951, 1952, and 1954, mostly during June, July, and August, on trees which ranged from apparently healthy specimens through individual trees in various stages of pole blight. Some of the trees were dominants in the stand (full light from above and some laterally) and some were codominants (full overhead light but little direct lateral light).

In planning the research, we had hoped to be able to introduce phosphorus 32 into the trees by normal absorption through the roots and to follow its translocation upward. From our preliminary studies it was apparent, however, that we would be unable to introduce sufficient activity into such large plants by normal means without seriously contaminating the site. Consequently we were forced to resort to injection directly into the stem.

To prepare the trees for injection, large metal or tar paper cups were cut and nailed or stapled to the stem after smoothing off the rough spots on the bark. The junctions between the cups and the boles were sealed with asphaltum. Three such metal cups were attached to the large trees and two to the smaller ones; in these cases placement of the cups included most of the circumference. The tar paper cups completely encircled the stem. To accomplish injection, the cups were nearly filled with a carrier solution of approximately pH 4 and 0.001 M with phosphate ion. Then the bark was pierced with a wood chisel to make a cut into the xylem approximately an inch deep, close to the junction of the tree and the cup. In no case was the tree completely girdled, however. After the

[1] Received revised manuscript June 19, 1959.

[2] This work was supported by a grant from the Atomic Energy Commission, Contract AT(45-1)-373 and by Special Research Project 11B of the University of Idaho.

[3] Present address: School of Forestry, Oregon State College, Corvallis.

[4] Present address: General Electric Co., Schenectady, N. Y.

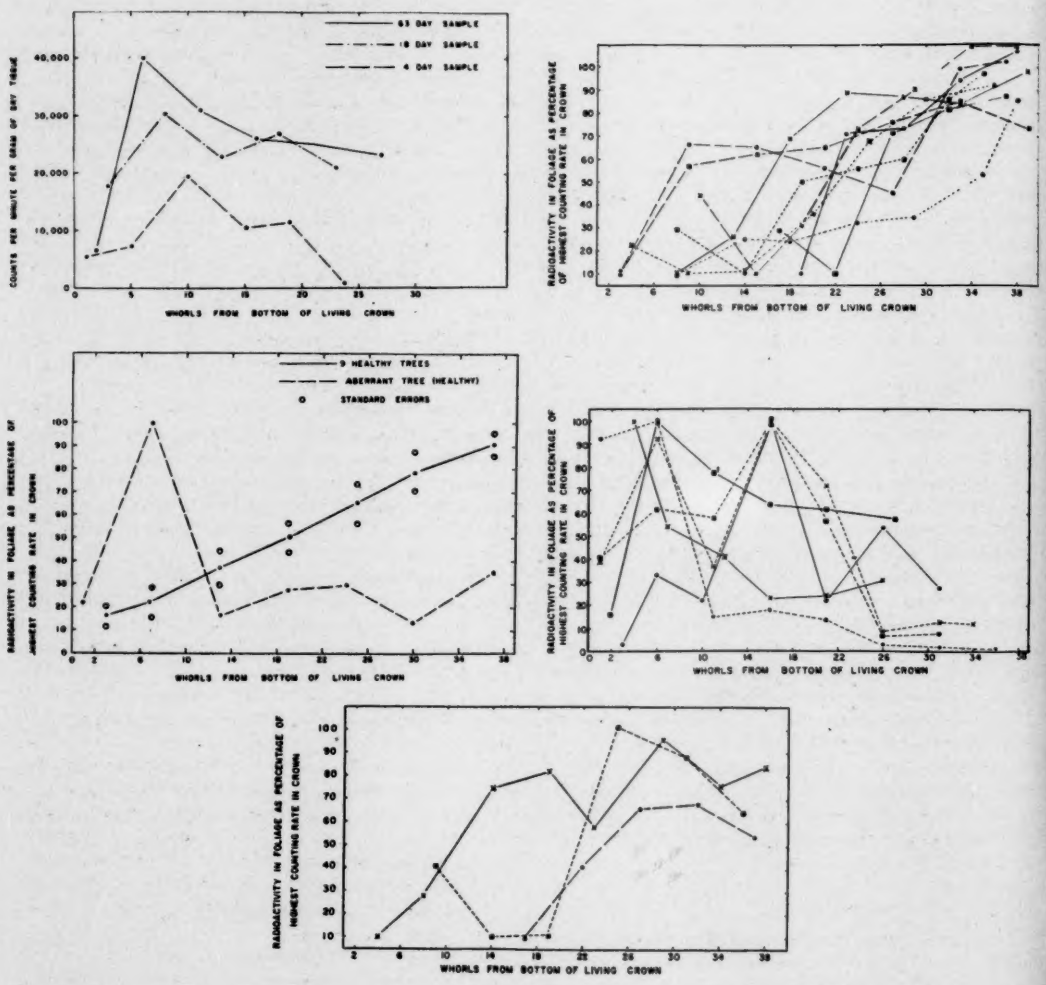

63 DAY SAMPLE
18 DAY SAMPLE
4 DAY SAMPLE
COUNTS PER MINUTE PER GRAM OF DRY TISSUE
WHORLS FROM BOTTOM OF LIVING CROWN
RADIOACTIVITY IN FOLIAGE AS PERCENTAGE OF HIGHEST COUNTING RATE IN CROWN
9 HEALTHY TREES
ABERRANT TREE (HEALTHY)
STANDARD ERRORS

FIG. 1 (*upper left*). Distribution of phosphorus 32 in foliage of a representative tree (pole blighted) at several sampling intervals.

FIG. 2 (*upper right*). Percentage distribution of phosphorus 32 in 1-year-old foliage in crowns of nine healthy trees 8 to 80 days after injection.

FIG. 3 (*middle left*). Average percentage distribution of phosphorus 32 in 1-year-old foliage in crowns of nine healthy trees 8 to 80 days after injection, with an aberrant healthy tree shown for contrast.

FIG. 4 (*middle right*). Percentage distribution of phosphorus 32 in 1-year-old foliage in crowns of pole blighted trees with well-developed symptoms in 8 to 80 days after injection.

FIG. 5 (*bottom*). Percentage distribution of phosphorus 32 in 1-year-old foliage in crowns of three early stage pole blighted trees 8 to 80 days after injection.

cutting operation, the phosphorus 32 in the form of 0.001 M phosphoric acid was pipetted into the cup to be taken up with the carrier solution. The trees received 2 to 9 liters of solution with from 0.9 to 5.8 millicuries of phosphorus 32; complete absorption from the cups took from 4 to 24 hours.

The foliage of the trees was sampled by climbing them with the aid of a ladder and cutting down the required branches. The whorls were numbered beginning with the bottom living whorl and the sampling was begun in the lower crown of the trees starting with the first reasonably symmetrical live whorl and continuing up the crown, sampling at five whorl intervals to the top of the tree. Usually it was impossible to get closer than the fifth whorl from the top because the upper branches were too small for support. To obtain needles from a given whorl, needles from the terminal shoot, outer twigs, and inner twigs were taken from each branch to insure a complete sampling. The 1-year-old needles were collected for analysis. Samplings of the foliage were made 2 to 5, 8 to 20, and 45 to 80 days after injection. Not all trees were sampled at all of these intervals, however.

In preparation for analysis, the needles were dried in a forced-draft oven at 70° C and then ground to a 40-mesh powder in a Wiley mill. Five grams of this material was compressed into a briquet according to the method of MacKenzie and Dean (5) and the leaf samples were radio-assayed in this form. A thin window Geiger-Mueller counter tube was used for the counting: the counting rates were corrected for coincidence loss, background, half-life (to time of injection), and sample weight, so that final data were in terms of counts per minute per gram of dry tissue. These counting rates were not corrected to 100 per cent geometry and so were not expressed in absolute numbers of counts. In addition, the total phosphorus for most samples was determined by wet digestion followed by the colorimetric molybdo-vanadate method (2).

## Results

For comparisons of the accumulation patterns of the trees, three different collection periods (2–5, 8–20, and 45–80 days after injection) were available for some trees, two were available for some, and only one for a few. Figure 1 shows the increase in accumulation with time for a representative pole blighted tree. The samples were not all made at the same whorls on the different collection periods. The increase in accumulation with time, came about apparently as the result of initial accumulation and then release of some phosphorus 32 in both xylem and phloem in its passage up the tree. Radioautographs of trunk sections taken 10 to 15 days after injection showed such accumulations as did the readings of a portable Geiger counter. Because the amount of accumulation in the foliage varied with time, the longest sampling period after injection was used for the computations. It was necessary to use data for 8-day collections for two trees because no other collections were made for them.

Counting rates in the foliage of the various trees varied a great deal, with the rate in one tree varying from 1,000 to 40,000 counts per minute per gram of tissue while the counting rate in another tree might vary from 50,000 to 250,000. A number of reasons account for these differences, the most prominent being the amount of foliage in a tree, the amount of radioisotope injected, and the differential amounts accumulated in the trunks of the trees on the passage upward. There was no indication of a difference in total foliar accumulation between diseased and healthy trees, however. The counts per minute per gram of tissue varied too widely between trees to show the relationships for all trees clearly on one graph, so they were transformed to percentage data with the highest counting rate in a given crown being designated as 100 %.

The distribution of phosphorus 32 in the crowns of the nine healthy trees is shown in figure 2. The general trends in these trees is for an increased accumulation proceeding up the crown. The crowns had different lengths and are plotted with the bottom as a common point of origin. The last whorl plotted for each tree is from 5 to 8 whorls from the extreme tip. If the plotting was done from the top as point of origin instead of the bottom, no apparent difference in accumulation pattern was evident.

The nine healthy trees were sufficiently similar in general accumulation that the data were transformed for pooling. To do this, the data of the longest crown was used as a standard and the other eight were exploded to crowns of equal size. The largest crown had 37 whorls and in a smaller-crowned tree with 26 whorls, for example, a whorl such as 16 would become whorl $(37/36 \times 16) = 23$, corresponding to whorl 23 on the tree with 37 whorls. The average distribution of phosphorus 32 by this pooling method is shown in figure 3. One healthy tree included neither in this group nor in figure 2 is shown separately in figure 3. This tree was completely different in behavior from the other healthy trees. The reasons are unknown although the tree may have been in an early, unrecognizable stage of pole blight.

The possibility that the aberrant healthy tree above might have been an early, unrecognizable pole blighted tree was suggested by the data of figure 4. Here the six known pole blighted trees with well-developed symptoms showed a trend of decreased accumulation in the upper, chlorotic portions of the crown as compared with the lower part of the crown. No different pattern emerges from this data if it is plotted with the top instead of the bottom of the crown as a base. In other words, there seems to be no tendency for maxima to appear at a different common morphological point in relation to the apex than in relation to the bottom of the crown. No attempt is made to show an average trend of data although it is clear that accumulation decreases with height in the crown.

In figure 5, three trees in what we took to be the early stages of the pole blight condition are shown. The accumulation patterns are more like those for

the healthy trees of figure 2. All 3 had only trunk symptoms of the disease with no crown symptoms.

In order to see if the accumulation of phosphorus 32 on a specific activity basis would show any different patterns, specific activities (counts/min/mg of total phosphorus) were calculated for all samples. The data are not presented here but they show the same trends of accumulation as are given in figures 2, 3, 4, and 5.

Another aspect investigated in two different years was the rate of movement of the phosphorus 32 in the stem after injection. To determine rates, the lower and middle crown foliage were sampled each day after the injection until activity significantly above background could be detected. The distance from this point to the point of injection was measured and the rate of movement was calculated, assuming straight grain in the tree. Generally two to four days were required for movement into the crown, but when movement was slow due to cloudy or rainy weather and high humidity, ten or more days were required for detectable quantities to reach the crown. The results of the measurements taken at several times are shown in table I. The data show that the rate of movement is more rapid in the pole blighted than in the healthy trees. One exception (32 cm/hr in the healthy compared with 23 cm/hr in the pole blighted) involved a tree in what appeared to be the very early stages of pole blight. In addition to the data presented in table I, qualitative data on the rate of movement of phosphorus 32 were obtained in other related studies. In these we found that four of five pole blighted trees injected had a considerable amount of phosphorus 32 in their crowns 4 days after injection while at the same time interval there was only a small amount of phosphorus 32 in the crowns of two of the five healthy trees and no detectable amount in the crowns of the three others. The most rapid rates of movement here would have been in excess of 25 cm/hour.

All of the figures on rate of movement presented above were averages for night and day movements over a number of days. The rates varied from 5 cm/hour or less when conditions for transpiration were poor to a rate in excess of 25 cm/hour when the conditions for transpiration were good. In summer, daytime rates alone must be considerably in excess of those reported here. Unpublished data obtained from our experiments with smaller trees, where the phosphorus 32 can move into the living crown in 1 day, show rates of movement in excess of 75 cm/hour. All of these rates are generally somewhat lower than those obtained by Moreland (6) for phosphorus 32 movement in loblolly pines.

It was mentioned earlier in the paper that the extreme tops of the pole blighted trees had yellow foliage and reduced growth. In order to determine to some degree whether the rate of movement was the same in the extreme tops of the pole blighted and healthy trees, some topping experiments were conducted. That is, the trees were climbed and the tops were sawed off 6 to 12 whorls from the top of the tree, the tops were dropped to the ground, and the cut ends were immediately placed in a container of water. After transport to a controlled area, the butts of these tops were placed in buckets containing several hundred microcuries of phosphorus 32 along with the carrier solution of 0.001 M ammonium phosphate. A healthy and a pole blighted top were placed in the bucket simultaneously and the bucket was placed in the sun. A total of four pole blighted and four healthy tops were treated in this way. From periodic sampling of the foliage and measurements of radioactivity and distance, it was determined that the rates of movement in two of the pole blighted trees were 5.7 cm and less than 1 cm/hour, while in the two corresponding healthy tops the rates of movement were 9.8 cm and 12.7 cm/hour. In the other two pairs of tops, accurate measurements of rate of movement were not made but it was obvious from the monitoring that was done that the rate of movement was more rapid in the healthy tops. These results suggest that there is less rapid movement in the extreme tops of the pole blighted than in the healthy trees, although for the tree as a whole the movement is more rapid in the pole blighted trees (table I).

TABLE I

RATE OF MOVEMENT OF PHOSPHORUS 32 IN HEALTHY AND POLE BLIGHTED TREES IN CM/HOUR

| HEALTHY TREES* | POLE BLIGHTED TREES * |
|---|---|
| 5.7 | 11.6 |
| 5.8 | 15.2 |
| 5 | 16 |
| 32 | 23 |
| 21 | 25 |
| 12 | 26 |
| 25 | 25 |
| 16 | 25 |

* Data between horizontal lines were taken at comparable times.

## DISCUSSION

One of the leading questions arising from the results reported above is that of the cause of the greater accumulation in one part of the crown than in another. Biddulph (1) has pointed out that accumulation is due to both transpiration and metabolic demands in various tissues in the plant. The top portions of the crowns of these trees are exposed to conditions very favorable for transpiration and this is where the greatest amounts of phosphorus 32 are concentrated in the healthy trees. Wright and Barton (10) have shown that phosphorus 32 accumulation may closely follow transpiration rates in leaves. The data for the distribution of phosphorus 32 (fig 2) are very much like what one would expect from the data on transpiration in tree crowns published by Pisek and Tranquillini (7).

On the other hand, the pole blighted trees show a definite trend for accumulation in the lower crown. Apparently the chlorotic foliage of the upper crown is unable to function normally in accumulating phosphorus 32. Probably this is due to a lowering in transpiration and metabolic processes which would normally favor accumulation.

The anomalous high concentration of total phosphorus in the tops of the pole blighted trees, mentioned in the introduction, is not explained by these data. They show an opposite trend, suggesting that the phosphorus may be deposited initially in the lower crowns of pole blighted trees and then transported to the tops later.

The phosphorus 32 data suggest that the transpiration rate in the lower crowns of the pole blighted trees was greater than in the healthy trees. On the other hand, in the upper crowns the transpiration rates were doubtless greater for the healthy trees. The net result is a more rapid movement in the pole blighted trees, at least to the lower crown. A transpiration rate in excess of normal has been commonly reported for other plant diseases in their progressive phases (4). It is also possible that the greater rate of movement in the pole blighted trees is due to a partial blocking of the conducting system in the bole; some of the conducting tissue is actually dead and resin-blocked in some of the pole blighted trees. Other things being equal, a reduction in conducting elements would cause an increase in rate of flow through the functioning elements.

We had hoped in this study to determine if the reduced phosphorus movement to the top of the crown in pole blighted trees might be one of the causes or the result of the condition. The data are not clear on this point, however. They suggest that the disturbance is the result and not the cause; trees apparently in the early phases of the disease have accumulation patterns more like healthy than like the more advanced pole blighted trees.

The data in figure 2 suggest an interesting possibility in the accumulation of materials in the upper crown during the summer. It seems probable that materials in the transpiration stream may be initially transported into regions of greatest transpiration, other things being equal. Hence, if the material, such as a phytotoxin, was to enter the transpiration stream at the same time of the year as the phosphorus was introduced, it would be deposited in largest amounts in the upper crown of a previously uninjured tree and there have its greatest effect. Some materials would be retransported out of the upper crown because we know from our analyses that the final total non-radioactive phosphorus distribution does not show the pattern given in figure 2, but is distributed fairly evenly throughout the healthy crowns. Abnormal materials such as phytotoxins might not be so easily retransported and the resultant injury could lead to the symptom picture which we see in pole blighted trees, with a deterioration of the upper crown of the tree and a progressively healthier appearance as one proceeds downward.

## Summary

Field experiments were performed to determine the distribution of phosphorus 32 after injection into pole-sized trees in healthy and in pole blighted condition. On the basis of activity per gram of leaf tissue, accumulations were largest in the upper crowns of the healthy trees and in the lower crowns of the pole blighted trees. Calculations on a specific activity basis gave no significant difference from the calculations of activity per gram of leaf tissue.

Taking the tree as a whole, the rate of phosphorus 32 movement was considerably faster in the pole blighted than in the healthy trees. When the extreme tops were severed, however, the rate in the severed tops was more rapid in the healthy than in the pole blighted trees.

## Acknowledgments

The authors wish to thank the U. S. Forest Service for providing field quarters and experimental material for this work at the Deception Creek Experimental Forest and at Clarkia Blister Rust Control Headquarters on the St. Joe National Forest.

## Literature Cited

1. Biddulph, O. 1951. The translocation of minerals in plants. In: Mineral Nutrition of Plants. Chap. 10. University of Wisconsin Press, Madison.
2. Bolin, D. W., and D. E. Stamberg. 1944. Rapid digestion method for determination of phosphorus. Ind. Eng. Chem. Anal. Ed. 16: 345.
3. Buchanan, T. S., G. M. Harvey, and D. S. Welch. 1951. Pole blight of western white pine: a numerical evaluation of the symptoms. Phytopathology 41: 199–208.
4. Gaumann, E. 1950. Principles of Plant Infection. Hafner Publ. Co., New York.
5. MacKenzie, A. J., and L. A. Dean. 1950. Measurement of $P^{32}$ in plant material by the use of briquets. Anal. Chem. 22: 489–490.
6. Moreland, D. E. 1950. A study of the translocation of radioactive phosphorus in loblolly pine (*Pinus taeda* L.). Jour. Elisha Mitchell Sci. Soc. 66: 175–181.
7. Pisek, A., and W. Tranquillini. 1951. Transpiration und Wasserhaushalt der Fichte (*Picea excelsea*) bei zunehurender Luft und Bodentrockenheit. Physiol. Plantarum 4: 1–27.
8. Wellner, C. A. 1948. A new disease threatens western white pine stands. Jour. For. 46: 294–295.
9. Wright, E. and D. P. Graham. 1952. Surveying for pole blight. Jour. Forestry 50: 680–682.
10. Wright, K. E. and N. L. Barton. 1955. Transpiration and the absorption and distribution of radioactive phosphorus in plants. Plant Physiol. 30: 386–388.

# UPTAKE AND UTILIZATION OF PHOSPHATE ASSOCIATED WITH RESPIRATORY CHANGES IN POTATO TUBER SLICES[1]

B. C. LOUGHMAN[2]

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

Respiration and salt absorption by slices of storage tissue have been extensively studied in recent years, with particular emphasis upon linkage between the two processes. In most investigations utilizing this type of material it is usual to bring the tissue into a state of steady metabolism before the start of the experiment. This is accomplished by subjecting freshly cut slices of tissue to exhaustive washing with water. The changes occurring during early stages of washing are, however, of great interest, but assessment of the factors governing this alteration of metabolism has rarely been attempted.

The specific respiration rate of freshly cut slices (1.0 mm thick) of potato may be five times that of the intact tuber and a further five-fold increase occurs in approximately 24 hours if the slices are kept in aerated water. Laties (11) has shown that the specific respiration rate of freshly cut slices is independent of thickness, whereas the development of the respiratory increment is controlled by the thickness. The respiration rate of neither fresh nor of aged slices is increased by increasing the oxygen content of the environment above that in air; Burton (1) has proven conclusively that tuber respiration is not limited by a shortage of oxygen within the tissue, since at normal storage temperatures the difference in oxygen concentration between the outside atmosphere and the center of the tuber is not great. The rise in respiration rate as a result of cutting must, therefore, be attributed to some cause other than the simple removal of a barrier to the diffusion of oxygen. Laties (11) has postulated that a volatile product of metabolism which imposes a restraint on respiration in the intact tuber diffuses out after cutting.

The demonstration by Hackett (7) that a marked change in the terminal oxidase characteristics occurs during the first 24 hours after cutting, is further evidence of the complexity of the processes involved in the development of increased respiratory power. Whereas 80 to 100 per cent of the respiration of fresh slices could be attributed to the cytochrome oxidase activity, the subsequent gain in respiration was found to be insensitive to carbon monoxide even though cytochrome oxidase activity could be demonstrated in mitochondria isolated from the tissue. The fact that an immediate rise in respiration rate of fresh potato slices can be brought about by adding $1 \times 10^{-5}$ M 2-4-dinitrophenol (DNP) suggested that the restraint on respiration is associated with systems involved in transfer of high energy phosphate.

A further distinction between the basal and induced respiration is illustrated by the fact that the former is insensitive to malonate whereas the latter is completely inhibited in the presence of this compound (9). It was assumed that two different types of metabolism are involved which differ in their dependence upon the turnover of phosphate. It was considered that the limitation of respiration in fresh tissue might be the result of reduced activity of the phosphate transferring systems, and as a consequence the phosphate metabolism of the two different types of tissue was examined from a number of points of view.

## MATERIALS

Potatoes obtained from local markets were washed and sliced into aerated ice water. All experiments were carried out with disks of tissue 9 mm in diameter and 1 mm thick. The varieties used were American Russet and English Majestic. After removing the adhering starch by three rinses in ice water, slices were stored in ice water for short periods prior to incubation in distilled water at 25° C or 30° C. The water was changed at 2 hour intervals for 12 hours, then at 8 hour intervals. The treatment was carried out in 1 liter Erlenmeyer flasks; the contents were agitated gently throughout in order to ensure adequate aeration. Respiration was measured by the standard Warburg method using ten 1.0 mm slices per vessel. The average fresh weight of ten slices was 0.65 g.

## METHODS

I. PHOSPHATE UPTAKE: Slices were threaded on stainless steel wire and separated by 2.0 mm diameter beads. Treatment was carried out at concentrations of $KH_2PO_4$ ranging from $10^{-6}$ M to $10^{-2}$ M at a pH of 4.5, and concentrations of $P^{32}$ ranging from 10 μc/l to 5 mc/l were used.

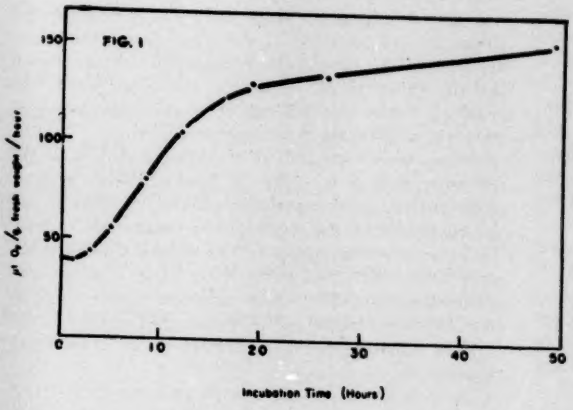
To distinguish active uptake from diffusion into the free space it was considered necessary to transfer slices to an equal volume of water at 0° C after the selected period of uptake to allow the free space phosphate to diffuse out again. The period in cold water was normally double the time allowed for uptake. On occasions the threaded slices were suspended in

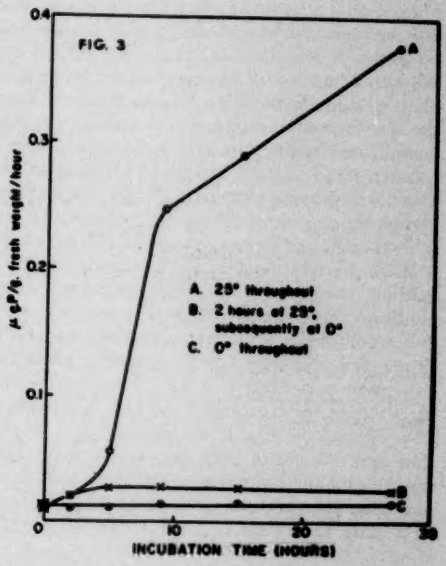
[1] Received August 18, 1959.

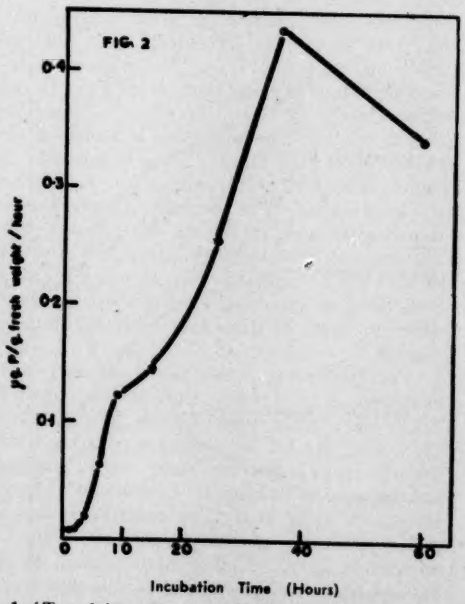
[2] Present address: Department of Agriculture, University of Oxford.

moist air, in order to prevent leakage of accumulated ions (3).

II. PHOSPHORYLATION: Tissue was extracted in the manner described in a previous paper (12). Samples of 0.65g fresh weight were normally extracted under ice cold conditions with 12 ml 0.1 *N* $HClO_4$. After short treatments with labelled phosphate, more than 90 per cent of the labelled compounds could be removed by two extractions. Extracting untreated slices by this procedure removed 60 per cent of the total phosphate, about half of this was inorganic. The initial chromatographic separations were carried out in *tert* butanol/water/picric acid (80ml/20ml/2.0 g) and further separations in propanol/ammonia/water (60ml/30ml/10ml).



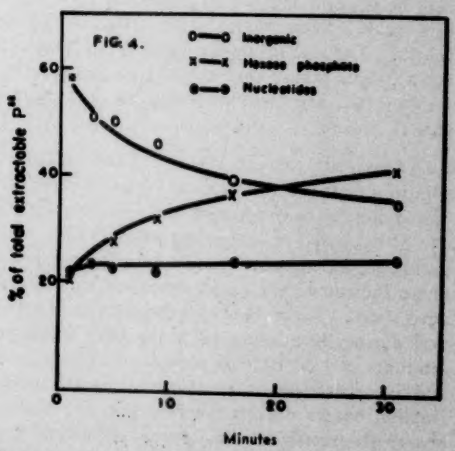




FIG. 1 (*Top, left*). Respiration of potato slices (1.0 mm thick) in aerated distilled water at 25° C.

FIG. 2 (*Bottom, left*). The course of phosphate uptake at pH 4.5 from $10^{-6}$ M $KH_2PO_4$ of 1.0 mm potato slices after incubation in aerated distilled water at 25° C.

FIG. 3 (*Top, right*). Phosphate uptake at pH 4.5 from $10^{-5}$ M $KH_2PO_4$ after incubation in aerated distilled water at different temperatures.

FIG. 4. (*Bottom, right*). The esterification of phosphate in 1.0 mm potato slices after 24 hours in aerated distilled water. Slices placed for 1 minute in labelled $10^{-5}$ M $KH_2PO_4$ prior to transfer to water at 25° C.

## Experimental Results

The typical rise in respiration obtained by incubating 1.0 mm slices in water at 25° C is shown in figure 1, where the autocatalytic nature of the process can be clearly seen. The corresponding curve for phosphate uptake capacity from $1 \times 10^{-6}$ M solution is shown in figure 2. After a 2 hour lag period a rapid rise occurs for 10 hours. The fluctuation in rate observed between 8 and 16 hours has been noticed on a number of occasions when low concentrations of phosphate are used, but no explanation can be given at this time. During the first 8 hours of incubation up to 10 per cent of the inorganic phosphate present in the tissue may be lost from the tissue; the accumulation studies with freshly cut slices are complicated by this fact. It is possible that this phosphate is lost from the cytoplasm rather than from the vacuole and could represent a large proportion of the cytoplasmic pool of inorganic phosphate.

An examination was made of the possibility that a short pre-treatment at 25° C would be sufficient to initiate changes such that the respiratory rise would continue subsequently at low temperature. Transfer to ice cold water effectively prevents any further rise in the capacity to take up phosphate at whatever stage this transfer is made (fig 3). The respiration values at 28 hours for treatments marked A, B, and C were 154, 45, and 34 $\mu$l/g fresh weight/hour, respectively. The development of both the capacities for phosphate uptake and for increased respiratory rate is dependent upon temperature, and slices can be maintained in a state equivalent to fresh tissue for several days if stored in water below 2° C.

The overall rise in the ability to absorb phosphate may be as great as 200-fold depending upon the initial low value which varies with different batches of tubers. The rise in uptake capacity increases rapidly for a longer period than the rise in respiration, suggesting that the two processes are not necessarily closely linked.

Esterification Pattern: After uptake from solutions of low concentration for periods of less than 15 minutes, up to 90 per cent of the absorbed phosphate can be extracted in cold dilute mineral acid. Investigation of the chemical nature of the absorbed phosphate indicated that rapid esterification occurred in aged slices. Figure 4 shows the pattern of esterification during 30 minutes in water after treatment for 1 minute in $1 \times 10^{-5}$ M phosphate at 25° C. Maximal incorporation of phosphate into the nucleotide fraction occurs during the first minute; a slow rise occurs thereafter in the hexose phosphate fraction. An overall esterification of 75 per cent of $P^{32}$-labelled phosphate occurs during the 30 minute period. During the 1 minute treatment no significant increase in the total amount of phosphate in the tissue occurs; the curves shown, therefore, represent the increase in specific activity of the designated fractions. The rate of esterification observed with aged tissue is of the same order as that found in actively growing young cereal roots (13).

The capacity for esterification of incoming phosphate rises with the ageing process. A 10 minute treatment in labelled phosphate ($1 \times 10^{-5}$ M) at 25° C was given to batches of slices removed at intervals from the incubation medium. Figure 5 shows that during the 10 minute experimental period fresh slices esterify 20 per cent of the incoming phosphate whereas after 24 hours at 25° C 85 per cent is esterified in 10 minutes. Since only small amounts of phosphate are taken up by the fresh tissue, the measurement of the esterification capacity lacks precision and the value of 20 per cent is the upper limit. In order to reduce the difficulties of this type of measurement, a different technique was used.

Slices were cut; half of them incubated at 25° C, the other half at 0° C for 24 hours. Slices at 0° C possessed the same metabolic activity as fresh tissue, the advantage of the treatment being that slices from the same tuber may be used for uptake studies at the same time. In addition, washing at 0° C appears to reduce the variability which exists among fresh slices from the same tuber. The terms aged and fresh will be used to distinguish slices treated at 25° C and 0° C, respectively.

A comparison of the absorbing capacity of fresh and aged slices was carried out under conditions where comparable amounts of labelled phosphate entered the tissue and leakage to the environment from the fresh slices was prevented. After treatment in labelled solution at 0° C for 15 minutes the slices were rapidly washed in three changes of water to raise the temperature to 25° C and to remove surface activity. The slices were then suspended in moist air to allow accumulation from the free space to proceed; samples then were assayed at intervals to determine the degree of esterification. The increase in esterification proceeds much more rapidly in aged slices (table I). The low rate of esterification in fresh slices reflects the slow rise in capacity to absorb inorganic phosphate from the free space and confirms earlier results obtained by assay of slices taken directly from labelled solution.

The behavior of fresh slices was more critically examined by a further experimental modification. Fresh slices were immersed in labelled phosphate ($1 \times 10^{-5}$ M) for 10 minutes at 0° C in order to fill the free space under conditions where metabolic accumulation was reduced to a minimum. After rapid rinsing in water at 0° C to remove surface activity the slices were suspended in moist air for 20 minutes at 25° C to allow accumulation to proceed in the absence of any leakage from the free space to the environment. At the end of the accumulation period the free space component was removed by washing in water at 0° C. This phosphate was all inorganic and represented about 50 per cent of the total. At this point the slices were returned to moist air at 25° C to follow the utilization of the absorbed phosphate. During the first hour less than 5 per cent

TABLE I

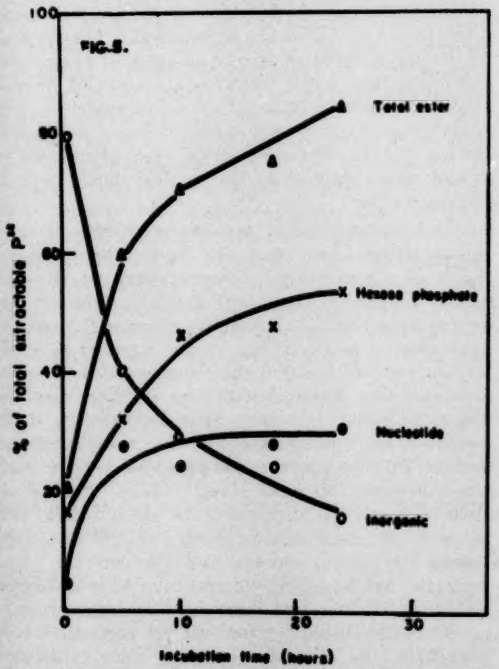
INCREASE IN ESTERIFICATION WITH TIME AT 25° C IN MOIST AIR AFTER FILLING FREE SPACE AT 0° C

| | TIME AT 25° C MIN | EXTRACTABLE PHOSPHATE CPM/MG FRESH WT | % ESTERIFICATION |
|---|---|---|---|
| Fresh | 2 | 143.0 | 12.6 |
| | 60 | 129.4 | 18.7 |
| | 180 | 157.4 | 30.1 |
| | 360 | 120.8 | 44.8 |
| | 660 | 121.0 | 58.8 |
| | 1,440 | 124.1 | 56.3 |
| Aged | 1 | 231.6 | 38.6 |
| | 2.5 | 252.0 | 48.1 |
| | 6 | 231.8 | 49.6 |
| | 12 | 200.3 | 56.7 |
| | 25 | 237.7 | 61.6 |
| | 60 | 221.9 | 55.0 |
| | 180 | 218.0 | 67.2 |

Slices placed for 15 minutes at 0° C in labelled solution ($10^{-5}$ M $KH_2PO_4$, 4μc/ml, pH 5.0) prior to rinsing and transfer to moist air.

esterification occurred and this value doubled during the next hour. After 24 hours, incorporation into both nucleotide and hexose phosphate occurred with an overall esterification of approximately 50 per cent indicating that the capacity of fresh slices to utilize absorbed phosphate increases with time. The results indicate that fresh tissue has a low capacity for esterifying incoming phosphate and that a marked increase in this capacity occurs with ageing. The respiration of aged potato slices is thus characterized by a very rapid turnover of phosphorus.

After an uptake period of 10 minutes in $1 \times 10^{-5}$ M $KH_2PO_4$ at 0° C followed by washing of aged slices for an equivalent time in water at the same temperature to remove the diffusible component, about half the phosphate remains in the tissue. About 40 per cent of this residue is removed if the tissue is washed in $1 \times 10^{-2}$ M $KH_2PO_4$ at 0° C and all this removable ion is inorganic orthophosphate. Thus, true accumulation of a small amount of phosphate occurs; this fraction also is inorganic. On raising the temperature to 25° C very rapid esterification occurs in the aged tissue. The process of accumulation appears to be separable from that of esterification. The inference is that phosphorylation is not essential



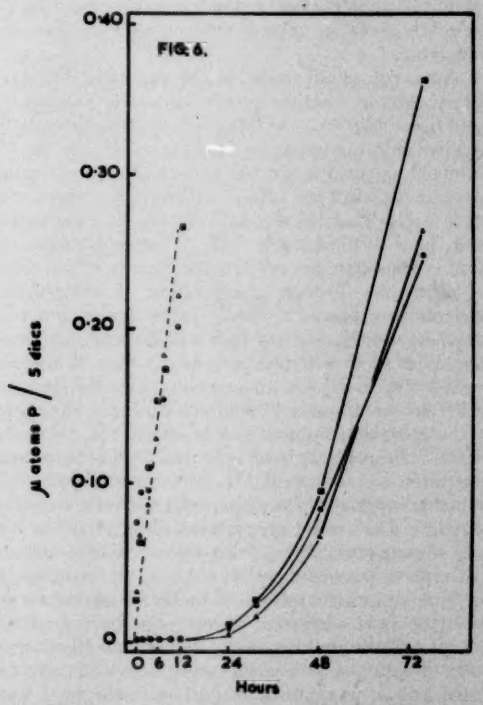




FIG. 5 (*Left*). The esterification capacity of 1.0 mm potato slices during incubation in aerated distilled water at 25° C. Slices treated for 10 minutes in labelled $10^{-5}$ M $KH_2PO_4$ prior to extraction with 0.1 M $HClO_4$.

FIG. 6 (*Right*). Phosphate uptake during incubation of 1.0 mm potato slices in $10^{-2}$ M (broken line) and $10^{-5}$ M (solid lines) phosphate solution at 16° C and pH 4.5. $KH_2PO_4$ (■ — ■), $NaH_2PO_4$ (▲ — ▲) and $LiH_2PO_4$ (● — ●).

to accumulation: it is assumed that phosphorylation occurs after accumulation. Only by working at 0° C can the rate of esterification be reduced sufficiently to distinguish between the two processes. Mechanisms of uptake can be suggested which involve the esterification at the cell surface of the incoming phosphate as an integral part of the absorption process, but it is more probable that the ion arrives in the cytoplasm in inorganic form, there to be incorporated into the metabolic systems of the tissue.

An interesting point is that whereas aged tissue at 0° C accumulates about four times as much $P^{32}$ as fresh tissue from $1 \times 10^{-5}$ M solution, yet, as has been shown earlier, the difference at 25° C may be up to 200-fold.

Assay of Cytoplasmic Pool Components: Measurements of the concentrations of the phosphorylated components turning over in the cytoplasmic pool are difficult, but preliminary assays have indicated that no large differences occur between fresh and aged tissue. There is evidence as shown above that uptake into the cytoplasm occurs prior to esterification and information on the size of the cytoplasmic pool of inorganic phosphate is highly desirable. Such estimates depend upon separation of vacuolar and cytoplasmic orthophosphate; the present experimental approach gives an approximate estimate of this distribution.

Apparent equilibration of the incoming $P^{32}$ with the nucleotide fraction occurs in a few minutes in aged tissue (fig 4). At this point the specific activity of the labile phosphate of isolated ATP can be determined as can the specific activity of the extracted inorganic phosphate. Two assumptions are now made: I. That all the ATP is in the cytoplasmic pool; none in the vacuole. II. That none of the incoming phosphate has reached the vacuole at this time.

Since the isotopic composition of cytoplasmic nucleotides achieves a steady value almost immediately, the specific activity (SA) of the terminal phosphates of ATP will be the same as that of the inorganic fraction. On this presumption the amount of $P^{31}$ required to satisfy this relationship with regard to the inorganic fraction can be calculated. The inorganic phosphate at issue is located in the cytoplasm. The difference between this value and the total extractable inorganic phosphate represents the vacuolar content. The second assumption is less probable, but even if some of the $P^{32}$ had already entered the vacuole this type of measurement should give an estimate of the maximal concentration of inorganic phosphate in the cytoplasm. Apparent isotopic equilibrium within the cytoplasmic pool can occur in less than 10 minutes under conditions where the phosphate entering the tissue makes no significant addition to the total concentration of phosphate within the tissue.

A value of 30 µg ATP per gram fresh weight is the upper limit obtained by spectrophotometric assay. In an experiment where apparent equilibrium was reached in 10 minutes, the nucleotide and inorganic fractions represented 29.1 per cent and 43.3 per cent of the extractable $P^{32}$, respectively. The corresponding concentrations of nucleotide phosphate and inorganic phosphate were approximately 3.0 and 42.2µg per gram fresh weight.

If it is assumed that the two terminal phosphate groups of ATP are equally labelled, whereas the $\alpha$ position is unlabelled, then the value for the concentration of nucleotide phosphate involved in the SA measurements is reduced to 2.0µg/g fresh weight, i.e. $SA = \frac{29.1}{2.0} = 14.6$. If the SA of these phosphate groups is the same as that of the inorganic phosphate, then the concentration of $P^{31}$ necessary to satisfy this requirement is $\frac{43.3}{14.6} = 3.0$. Consequently, this value of 3.0µg represents the inorganic phosphate contained in the cytoplasmic pool. Since the total inorganic phosphate of the tissue is 42.0µg/g fresh weight, then it appears that more than 90 per cent of this phosphate is contained in the vacuole. If, as has been shown earlier, 10 per cent of the tissue phosphate can be lost during the early stages of washing, this amount possibly represents the greater portion of the cytoplasmic inorganic component. Refinement of the techniques used here is necessary before accurate determination of the distribution of phosphate between vacuole and cytoplasm can be obtained. The presence of uridine phosphates as contaminants of the isolated ATP has been noted and accurate assay of these is important before precise values can be obtained. The assumption is tentative that no $P^{32}$ enters the vacuole during the equilibration period, but calculation of cytoplasmic phosphate by this method should give the maximal value.

That the increase in respiration brought about in ageing tissue is associated with increased enzyme synthesis is a possibility. Concentrations of chloramphenicol shown by Gale and Folkes (6) to inhibit protein synthesis in *E. coli* have no effect on the respiration rise of potato slices. After ageing slices for 24 hours in 0.01 per cent chloramphenicol the respiration rate was identical with that of slices aged for the same period in water. The activities of starch phosphorylase, phosphoglucomutase, phosphohexoisomerase, amylase, apyrase, and phosphatase were measured in fresh and aged slices. These enzymes are involved in the mobilization of the major reserve substrate in the tissue, but no marked change in activity during the ageing process could be detected. This suggests that the glycolytic system at least undergoes no extensive alteration during the ageing process.

The relationship between rate of accumulation of phosphate and concentration was widely different for fresh and aged slices. Table II shows the comparative rates of uptake at $1 \times 10^{-5}$ M and $1 \times 10^{-2}$ M $KH_2PO_4$. After a 10 minute absorption period the slices were washed for an equivalent time in water or $1 \times 10^{-2}$ M $KH_2PO_4$.

TABLE II

RATE OF PHOSPHATE UPTAKE BY FRESH AND AGED 1.0 MM SLICES

| | Uptake rate (μg/hr/g fresh wt) | | | | | |
|---|---|---|---|---|---|---|
| | Fresh | | | Aged | | |
| | $10^{-5}$ M | $10^{-2}$ M | $10^{-2}$ M / $10^{-5}$ M | $10^{-5}$ M | $10^{-2}$ M | $10^{-2}$ M / $10^{-5}$ M |
| Washed in $H_2O$ | 0.0172 | 8.52 | 495 | 0.728 | 23.06 | 31.7 |
| Washed in $10^{-2}$ M $KH_2PO_4$ | 0.0102 | 7.65 | 750 | 0.843 | 22.18 | 24.8 |

Slices treated for 10 minutes at 25° C in labelled $10^{-5}$ M or $10^{-2}$ M $KH_2PO_4$ at pH 4.5 followed by 10 minutes in water or $10^{-2}$ M $KH_2PO_4$ at 0° C.

Whereas aged slices at $10^{-5}$ M accumulated 40 to 80 times as much phosphate as fresh material the corresponding ratio at $10^{-2}$ M was 2.7 to 2.9. The manner in which this ratio alters with concentration is shown in table III.

The respiration of the fresh tissue remains unaltered over this range of $KH_2PO_4$ concentration; similar uptake rates are obtained if the potassium is replaced by sodium or lithium though the latter ion at a concentration of $10^{-2}$ M causes the uptake of phosphate to cease completely after 24 hours. Figure 6 shows the uptake of phosphate during the ageing of slices in $10^{-2}$ M and $10^{-5}$ M phosphate solution in the presence of $K^+$, $Na^+$, and $Li^+$ as the accompanying cations. High concentrations of lithium have been shown by Laties (10) to prevent the normal respiration rise after cutting.

Fresh slices absorb phosphate readily from solutions of high concentration. They also exhibit an ability for esterification equal to aged slices. This observation is in agreement with the results of Calo, Marks, and Varner (2), who consider the phosphorylative capacity of fresh and aged slices to be identical in $10^{-2}$ M solution. That the metabolism of fresh tissue is not limited by phosphate supply is indicated by the fact that the respiratory rate is not increased when slices are immersed in $10^{-2}$ M $KH_2PO_4$.

TABLE III

EFFECT OF PHOSPHATE CONCENTRATION ON UPTAKE CAPACITY OF FRESH AND AGED 1.0 MM SLICES

| | Uptake rate (μg/hr/g fresh wt) | | |
|---|---|---|---|
| M $KH_2PO_4$ | Fresh | Aged | Ratio Aged / Fresh |
| $10^{-6}$ | 0.0024 | 0.216 | 89.6 |
| $10^{-5}$ | 0.012 | 1.30 | 107.5 |
| $10^{-4}$ | 0.112 | 2.48 | 22.35 |
| $10^{-3}$ | 1.32 | 5.92 | 4.53 |
| $10^{-2}$ | 13.68 | 27.80 | 2.02 |

Slices placed for 15 minutes at 30° C in labelled solution (pH 5.0) followed by 30 minutes in water at 0° C.

Whereas fresh slices esterify only about five per cent of the phosphate absorbed in 10 minutes from $10^{-5}$ M solution, up to 70 per cent may be esterified at $10^{-2}$ M. It is clear that the rapid accumulation of phosphate by fresh slices at high concentration is accompanied by a high rate of esterification, but it has not been established whether the processes are readily separable or not.

The data of Hagen and Hopkins (8) suggest that two sites are involved in the accumulation of phosphate, one for the $HPO_4^{--}$ ion and the other for the $H_2PO_4^-$ ion. Extension of their approach to cation uptake by Fried and Noggle (4) led them to conclude that the accumulation of monovalent cations proceeds at two sites, one of which operates at high concentrations and the other at low. The results presented here suggest that in fresh tissue the site operating at low concentrations is non-functional whereas the other is operating normally. Thus, ageing of the tissue is intimately associated with development of the low concentration site.

## DISCUSSION

The results obtained at low phosphate concentrations indicate that the phosphorolytic capacity of aged slices is very high, whereas that of freshly cut slices is almost negligible. Under conditions of low phosphate supply, therefore, it is reasonable to assume that the respiration developed during ageing at 25° C is in fact characterized by a rapid turnover of phosphate, whereas the basal respiration is characterized primarily by non-phosphorolysis. The fact that active uptake occurs in fresh tissue in the presence of high concentrations of phosphate can be explained by assuming that two different sites of uptake are involved, differing markedly in the concentrations at which they become saturated. Kinetic treatment of the results in the manner described by Hagen and Hopkins (8) gives confirmation of this view, although detailed investigation of the characteristics of the sites is premature at this point. The results are consistent with the view that the gain in respiration associated with ageing is linked with the creation of a site of uptake operating solely at low concentration.

Having shown that $H_2PO_4^-$ and $HPO_4^{--}$ ions were absorbed at different sites in barley roots, Hagen and Hopkins (8) calculated the dissociation constants of the two ion-carrier complexes and found them to be $4 \times 10^{-5}$ M and $5 \times 10^{-10}$ M, respectively. A similar pattern for uptake sites operating at high and low concentrations of single monovalent cations has been reported by Fried and Noggle (4). It seems that this phenomenon may be of general occurrence.

One of the possibilities arising from studies with aged slices at 0° C is that phosphate is actually accumulated by the cytoplasm in inorganic form and that the process involving rapid esterification is a completely separate stage. The rapidity with which the incoming phosphate comes into apparent equilibrium with the nucleotide fraction, and the high proportion of phosphate esterified suggests that, compared to the cell as a whole, the inorganic phosphate content of the cytoplasm is low. The basis for measuring this distribution has already been discussed. It appears that refinement of the techniques used could furnish reliable values. Where the synthesis of nuclear components is studied by means of externally applied radioactive inorganic phosphate as in nucleic acid synthesis, knowledge of the phosphate concentration in the cytoplasmic environment would appear to be a valuable aid to the interpretation of the results obtained (14).

The observation (6) that the capacity of aged slices to absorb phosphate, is inhibited by carbon monoxide in the dark while the respiration remains unaffected and that the inhibition is light reversible, suggests strongly that the portion of respiration associated with phosphate uptake is mediated by cytochrome oxidase, whereas the overall respiration is not. The study of the relationship between the induced respiration and the new terminal oxidase system and the identification of the latter appears to be of first importance in elucidating the problems involved in the control of storage tissue metabolism.

## Summary

I. The increase in respiration observed when freshly cut potato slices are incubated in aerated distilled water for 24 hours at 25° C is associated with a change in the ability to utilize phosphate from solutions of low concentration.

II. The capacity for uptake rises by more than 100-fold, and a 20-fold increase in the ability to esterify the accumulated phosphate can occur during the same period.

III. Fresh and aged slices show little difference in their capacity to accumulate and esterify phosphate from solutions of high concentration.

IV. The increase in respiration during ageing appears to be associated with the synthesis of a cellular component concerned in the accumulation of phosphate at low concentration.

V. Accumulation and esterification seem to be separate processes. Incorporation of phosphate into organic forms is not necessarily essential for uptake into the cell.

VI. Respiration of fresh tissue is possibly non-phosphorolytic in nature, whereas that of aged tissue is characterized by a rapid turnover of phosphate.

VII. The greater part of the inorganic phosphate in the cell appears to be stored in the vacuole and takes no part in the steady state metabolism of the cell.

## Acknowledgments

The author wishes to record his appreciation for the advice and counsel given by Dr. George Laties at whose suggestion the work was undertaken. The financial help of the National Science Foundation is gratefully acknowledged.

## Literature Cited

1. Burton, W. G. 1950. Studies on the dormancy and sprouting of potatoes. 1. The oxygen content of the potato tuber. New Phytologist 49: 121–134.
2. Calo, N., J. Marks, and J. E. Varner. 1957. Respiratory metabolism of aerated potato disks. Nature 180: 1142.
3. Epstein, E. 1955. Passive permeation and active transport of ions in plant roots. Plant Physiol. 30: 529–535.
4. Fried, M. and J. C. Noggle. 1958. Multiple site uptake of individual cations by roots as affected by hydrogen ion. Plant Physiol. 33: 139–144.
5. Gale, E. F. and J. P. Folkes. 1955. The assimilation of amino acids by bacteria. 20. The incorporation of labelled amino acids by disrupted staphylococcal cells. Biochem. Jour. 59: 661–675.
6. Griffiths, S. K. and D. P. Hackett. 1957. Respiratory mechanisms associated with inorganic phosphate uptake by potato tuber tissue. Plant Physiol. suppl.: 32 xlvi.
7. Hackett, D. P. 1956. Studies on the "wound respiration" of potato tuber tissue. Plant Physiol. suppl.: 31 xl.
8. Hagen, C. E. and H. T. Hopkins. 1955. Ionic species in orthophosphate absorption by barley roots. Plant Physiol. 30: 193–199.
9. Laties, G. G. 1959. The nature of the respiratory rise in slices of chicory root. Arch. Biochem. Biophys. 79: 364–377.
10. Laties, G. G. 1959. The development and control of coexisting respiratory systems in slices of chicory root. Arch. Biochem. Biophys. 79: 378–391.
11. Laties, G. G. 1957. Respiration and cellular work and the regulation of the respiration rate in plants. In: Survey of Biological Progress Vol. III. Pp. 215–299. Academic Press, New York.
12. Loughman, B. C. and R. P. Martin. 1957. Methods and equipment for the study of the incorporation of phosphorus by intact barley plants in experiments of short duration. Jour. Exp. Bot. 8: 272–279.
13. Loughman, B. C. and R. S. Russell. 1957. The absorption and utilization of phosphate by young barley plants. IV. The initial stages of phosphate metabolism in roots. Jour. Exp. Bot. 8: 280–293.
14. Ts'o, P. O. P. and C. S. Sato. 1959. Synthesis of ribonucleic acid in plants. II. Phosphate-$P^{32}$ incorporation into ribonucleic acid of the pea epicotyls. Exp. Cell. Research 17: 237–245.

# PHOTOSYNTHESIS AND RESPIRATION IN DEVELOPING FRUITS
## I. $C^{14}O_2$ UPTAKE BY YOUNG ORANGES IN LIGHT AND IN DARK[1]

R. C. BEAN AND G. W. TODD[2]

DEPARTMENT OF PLANT BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, RIVERSIDE

The effect that photosynthesis in a green fruit may have on the growth of that fruit has not been extensively studied. In another paper of this series (Todd, Bean, and Propst, unpublished results) a study was made of the magnitude of photosynthesis in cucumbers, lemons, oranges, and avocados at different ages and under varying conditions. Photosynthesis in these fruits could completely compensate for respiratory loss of carbon dioxide, under some conditions. With normal growth situations, however, it may be assumed that photosynthesis in the fruit itself does not supply a large proportion of the material needed for growth of the fruit.

Assimilation of carbon dioxide might also affect the type of materials produced and stored within a given tissue. Even though photosynthetic assimilation might account for a relatively small percentage of the total stored material, the compounds formed in fixation of carbon dioxide might have a strong effect on the quality of the fruit. To help determine what role carbon dioxide assimilation may play in formation of storage products, a study has been made of the materials formed by fixation of carbon dioxide in young fruit in the light and in the dark, using radioactive tracers. The present paper gives some of the results found with young oranges. It appears from some of these results that carbon dioxide fixation in the dark by juice vesicles of these small oranges may be involved extensively in the formation and storage of the organic acids of the orange juice.

### MATERIALS AND METHODS

A single, intact Valencia orange (13.4 g – 15.7 g) was placed in each of the two chambers of the carbon dioxide fixation apparatus pictured in figure 1. Each chamber also contained 100 mg samples of each of the three major tissues of the orange, the flavedo (outer, green layer of the peel), albedo (inner, white peel layer), and the juice vesicles. The vesicles, or juice sacs, were prepared by carefully teasing them loose from the carpellary membrane. Flavedo and albedo were prepared by dividing diametric sections of the fruit (about 1.5 mm thickness) into the two tissues. The chambers were immersed in a 25° C water bath and illuminated from below by fluorescent lamps (ca 1,000 ft-c). After reducing pressure in the system slightly, carbon dioxide was generated by adding lactic acid to $BaC^{14}O_3$ (3.4 mg, 82 μc/mg). The circulating pump kept a constant flow of gas through the apparatus during the experimental period at a rate such that the gas should completely recycle about four times per minute (flow rate 1200 ml/min, apparatus volume 280 ml). Thus, both light and dark samples were exposed to identical atmospheres. The dark flask was wrapped with black plastic tape to keep light out.

At the end of the experimental period (220 min) the air from the chambers was withdrawn rapidly into an evacuated 2,000 ml flask containing standardized, carbon dioxide free sodium hydroxide. Subsequent titration of the alkali allowed a determination of the final carbon dioxide concentration in the system. Each of the tissue samples was dropped directly into separate vessels of rapidly boiling ethanol. The intact fruits were rapidly frozen in crushed, frozen $CO_2$. They were allowed to warm to −20° C, separated into the flavedo, albedo, and pulp, and each tissue was inactivated and extracted in boiling ethanol.

After centrifuging and decanting the initial alcohol extracts, extraction of each of the samples was continued by adding a small amount of water to each sample and grinding while heating over steam. After

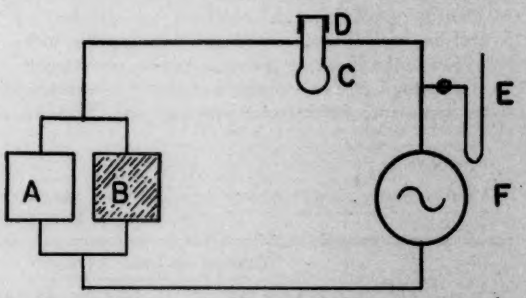



FIG. 1. Carbon dioxide assimilation apparatus. A. Light chamber. B. Dark chamber. Wrapped with plastic electrical tape. C. $C^{14}O_2$ generating chamber containing $BaC^{14}O_3$. D. Ampoule cap. To allow addition of lactic acid to $BaC^{14}O_3$ with a syringe. E. Manometer. F. Circulating pump. Chambers were immersed in a 25° C water bath and illuminated from below by fluorescent lamps.

Total volume of the apparatus air space was 280 ml.

[1] Received September 8, 1959.

[2] Present address: Department of Botany and Plant Pathology, Oklahoma State University, Stillwater.

the samples were fairly well macerated, alcohol was added to give a concentration of 80 per cent and the samples boiled for a few minutes. This process was repeated several times with centrifuging and decanting between each repetition. The residues were subjected to continuous extraction by alcohol in a Bailey-Walker apparatus to remove the last traces of soluble material. This latter step removed only a very small percentage (less than 1 % of the total alcohol extractable activity but it was required to obtain consistent results on the activity of the insoluble residues.

All the extracts from each sample were combined and diluted to known volumes. Aliquots were taken and plated directly on stainless steel planchets for determination of total activity fixed in each tissue. Larger aliquots were evaporated to dryness at 30° to 40° C to obtain samples for chromatographic analysis. These samples were redissolved in known amounts of water and applied to filter paper. One dimensional chromatograms were prepared with water-saturated phenol, butanol-acetic acid-water (52:13:35), or butanol-ethanol-water (52.5:32:15.5). Two dimensional chromatograms were also prepared with the first two solvents. The radioactive compounds were located by radioautography. Counting of activity was done directly on the chromatographic paper, using previously determined conversion factors to obtain figures comparable to those found with counting on steel planchets.

Labeled compounds found on the chromatograms were tentatively identified by chromatographic positions and by reaction to spray reagents (1 % *p*-anisidine hydrochloride in butanol for sugars, 0.1 % ninhydrin in acetone as a dipping agent for amino acids, 0.04 % bromcresol green in alcohol for organic acids). For confirmation of the identity of glucose, the glucose area was eluted and treated with fungal glucose oxidase and the reaction mixture rechromatographed to identify gluconic acid. Sucrose was checked by hydrolysis with invertase and chromatographic identification of the resulting monosaccharide components. Rechromatography of the reaction products resulting from treatment of the eluted fructose area with calcium hydroxide showed a normal distribution of activity between the epimers, fructose, mannose, and glucose. Amino acids and organic acids were mixed with authentic samples of the suspected acid and rechromatographed in other solvents. Coincidence in several solvents of the radioactive spot with the colored spot resulting from the indicator reaction was considered adequate identification for the purposes of this study. The amount of activity lost upon treatment of the radioactive spots with ninhydrin also contributed to confirmation of the identity of the amino acids. Organic phosphates were in such low concentration that identification was made only upon the original chromatographic characteristics.

The alcohol-insoluble residues were ground finely and alcohol suspensions of the powder were plated for counting. Approximate absorption factors were obtained on the assumption that the residue powder would have the same absorption as a similar thickness and weight of filter paper.

All radioactivity determinations were made using a Nuclear-Chicago, D-47 gas-flow tube with a thin end window. For counting on paper chromatograms, this tube, without shielding, was supported on a ring about 2 mm from the paper.

To determine the relative distribution of activity in the carboxyl groups of malic acid, the $\beta$-carboxyl was obtained as carbon dioxide by treatment with *Lactobacillus arabinosus* (9). Both carboxyl groups were obtained by adapting the method of Friedman and Kendall (5). The $\alpha$-carboxyl activity was taken as the difference between the two determinations.

Carbon dioxide concentrations during the experiment were estimated from the initial carbonate introduced and titration of the trapped carbon dioxide at the end of the experiment.

## Results and Discussion

The total fixation of carbon dioxide per unit weight of total solid material in the alcohol-soluble and alcohol-insoluble fractions of all tissues of the fruit is compiled in table I. Preliminary experiments indicated that fixation was relatively uniform when ex-

Table I

$C^{14}$-Activity in Alcohol-Soluble and Alcohol-Insoluble Fractions of Orange Tissues Exposed to $C^{14}O_2$ [1, 2]

| | Alcohol-soluble extract[3] | | | | Alcohol-insoluble residue | | | |
|---|---|---|---|---|---|---|---|---|
| | Intact fruit | | Tissue preparation | | Intact fruit | | Tissue preparation | |
| | Light | Dark | Light | Dark | Light | Dark | Light | Dark |
| Flavedo | 34,700 | 1,540 | 54,000 | 3,700 | 15,400 | 280 | 6,670 | 490 |
| Albedo | 3,400 | 1,150 | 2,500 | 1,950 | 235 | 160 | 280 | 280 |
| Vesicle | 2,580 | 2,500 | 13,600 | 16,500 | 284 | 360 | 2,580 | 1,680 |

[1] Initial carbon dioxide concentration was about 0.2 %; final 2.5 %.

[2] Activity is given as cpm/mg of the dried soluble solids or of the alcohol-insoluble residue. Counting was done to within 3 % statistical error.

[3] Total activity in the various extracts from intact fruits: Light ($10^5$ cpm): Flavedo, 165; albedo, 18.0; vesicles, 11.0; Dark ($10^5$ cpm): Flavedo, 6.9; albedo, 5.7; vesicles, 7.4.

pressed on the basis of unit solid material for other sizes of fruit but total activity would vary greatly. Total activity in the extracts from tissues of the intact fruits is given as a footnote to table I. The $C^{14}$ activity found in preparations of the illuminated flavedo is very much higher than that found in the dark flavedo or in any other tissue. The activity of extracts from the photosynthesizing flavedo range from 14 to 23 times the activity of the dark flavedo and similar ratios (14–55 fold) are found in the alcohol-insoluble material. This indicates that the green flavedo is capable of a respectable photosynthetic assimilation rate and agrees with previous determinations on the intact fruits (Todd, Bean, and Propst, unpublished results).

Illumination causes a rather large increase in activity in the albedo tissues of the intact orange (3:1 ratio as shown in table I). The difference between light and dark fixation is negligible with sections of albedo. The fact that illumination causes increased activity only in the albedo of the intact fruit and not in the albedo tissue sections would make it appear that the major part of the increase in albedo activity in the intact fruit may be due to translocation of photosynthate into the albedo from the highly active flavedo tissues. This conclusion is further supported by consideration of the activities found in individual components of the tissues (table II and discussion below).

The juice sacs show essentially no difference in total assimilation of activity in the light and the dark, whether as an intact fruit or tissue preparation (table I). There is a strong difference between assimilation in the vesicles of the intact fruit and in the isolated vesicles. This might be expected since the respiratory carbon dioxide from surrounding tissues in the intact fruit would greatly dilute the activity of the carbon dioxide reaching the vesicles. Despite showing no significant photosynthetic activity, the vesicles were capable of a very high degree of $C^{14}O_2$ fixation. The isolated juice sacs were able to assimilate from 25 to 30 per cent of the activity fixed per unit of soluble material, by the photosynthesizing, isolated flavedo. In the dark intact fruit, the unit activity of the vesicle extract is considerably higher than that of either of the two tissues which completely surround it. Comparison of the total activities fixed in extracts from different tissues of intact fruit (footnote to table I) further accents the ability of the vesicles to accumulate carbon dioxide. The vesicles in the dark were found to have fixed a total of $7.4 \times 10^5$ cpm while the flavedo only contained $6.9 \times 10^5$ cpm. These factors would suggest that the vesicles may contain a highly efficient system for exchange or dark fixation of carbon dioxide.

Table II shows some of the differences found in the fixation of activity into the individual components of the alcohol extracts of various tissues. The sugars are highly labeled in the photosynthesizing flavedo, as would be expected in the primary photosynthesizing tissue. Sucrose is found with relatively high activity in the albedo of the intact fruit but only a very small amount of activity is found in sucrose in the illuminated, isolated albedo. This tends to support the conclusion above that the major increase in activity of the albedo of the intact, photosynthesizing fruit is due to translocation of activity (in the form of sucrose) from the flavedo. A very slight amount of activity is found in sucrose in the illuminated, isolated vesicles indicating that even these tissues may be capable of a very limited amount of photosynthesis at this stage of development.

Aspartic acid, serine, and malic acid are all fairly strongly labeled in the dark. Little effect of light is found on the amount of activity in these compounds except in the photosynthesizing flavedo where malic acid and serine show fairly strong increases in the light. In other plants, it has been shown that malic

TABLE II

DISTRIBUTION OF $C^{14}$ ACTIVITY IN COMPONENTS OF ALCOHOL-SOLUBLE EXTRACTS FROM ORANGE TISSUES[1]

| | Intact fruit | | | | | | Tissue preparations | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Flavedo | | Albedo | | Vesicles | | Flavedo | | Albedo | | Vesicles | |
| Component | Light | Dark | Light | Dark | Light | Dark | Light | Dark | Light | Dark | Light | Dark |
| Glucose | 1,020 | ... | ... | ... | ... | ... | 1,290 | ... | ... | ... | ... | ... |
| Sucrose | 10,400 | ... | 1,140 | ... | ... | ... | 9,870 | ... | 159 | ... | 146 | ... |
| Fructose | 810 | ... | ... | ... | ... | ... | 860 | ... | ... | ... | ... | ... |
| Alanine | 100 | ... | 5 | ... | + | + | 330 | ... | + | ... | ... | ... |
| Glutamic Acid | 1,200 | + | 37 | + | + | + | 610 | 13 | + | ... | 37 | + |
| Aspartic Acid | + | 90 | 10 | 55 | 275 | 190 | 160 | 40 | 35 | 23 | 182 | 209 |
| Serine | 180 | 65 | 45 | 100 | 200 | 185 | 120 | 70 | 135 | 110 | 146 | 101 |
| Malic Acid | 860 | 435 | 45 | 57 | 325 | 355 | 950 | 420 | 141 | 120 | 1,210 | 1,340 |
| Citric Acid | 30 | ... | 30 | 52 | 460 | 630 | 33 | 20 | ... | ... | 475 | 445 |
| Other Acids[2] | + | + | + | + | 160 | 160 | 67 | 67 | 88 | + | 273 | 283 |
| Phosphate Esters | + | ... | ... | ... | ... | ... | 87 | 67 | ... | ... | 30 | ... |

[1] Activity given throughout as counts per minute per milligram of dried soluble extract. Activity was negligible or too low for measurement where no figure is given.

[2] Succinate and fumarate were lost by running off the edge of the paper. Generally they were of low activity.

acid is not directly concerned with photosynthetic assimilation of $CO_2$ but an increase in rate of labeling of malic acid in the light occurs as an indirect effect of photosynthesis (3) since compounds in equilibrium with malic acid are becoming labeled. Citric, glutamic, glycolic, glyceric, succinic, and fumaric acids are also labeled in the dark. The distribution and total amount of activity in these compounds varied from tissue to tissue but all the same compounds are labeled throughout, except for citric acid which seems to show some erratic behavior in the albedo and flavedo. Acid fractionations have shown that citric acid is in low concentration in peel tissues although it comprises the major acid of the vesicles at this developmental stage.

A number of factors indicate that the dark products found here must be formed within each tissue rather than by extensive translocation from one tissue to another. Each tissue, individually, is capable of fixing a significant amount of activity through dark reactions with carbon dioxide as shown by the tissue preparation data in table I. In the intact fruits, despite the increase and redistribution of activity in the flavedo caused by photosynthesis, there is virtually no effect on the activity found in the vesicles. Even in the albedo of the intact fruit the changes due to photosynthesis appear to be restricted to carbohydrate components. A highly efficient transport system would be required to build up the amount of activity found in the vesicles of the intact fruit during the dark reaction if it were assumed that the labeled compounds of the vesicles arose by reactions with carbon dioxide in the peel followed by movement into the vesicles for storage. Such transport is rather unlikely in view of the lack of apparent movement of the labeled sugars from the peel into the vesicles during photosynthesis. The high unit activity of the dark vesicle extracts in comparison with those from the other tissues also argues against such a transport effect. Determinations of the distribution of activity in the carboxyl groups of malic acid from the vesicles of intact fruits showed that the $\beta$-carboxyl contained about 75 per cent of the activity whether the fruit was photosynthesizing or in the dark. This lack of effect of light on the distribution further confirms an independent fixation in the vesicles.

With only a dark fixation mechanism in operation, the vesicles have accumulated a strikingly large amount of activity, as shown in the two tables. The high accumulation of carbon dioxide could be indicative of a possible mechanism for the formation of a portion of the acids which are stored in the endocarp of citrus fruits. In some succulent plants a net uptake of carbon dioxide may occur in the dark to give rise to an increase in organic acids (4, 12, 13, 14). Fixation of carbon dioxide in the dark appears to be a general reaction for plants (7, 8) although only a few plants carry it on to such an extent as to produce a measurable uptake. In succulents the dark uptake appears to depend upon utilization of sugars to form acceptor units. Sugars are depleted during the dark reaction (13) while the acids increase. The reaction of phospho-enol pyruvate with carbon dioxide (11) appears to be one of the reactions responsible for the dark fixation in succulents and other plants (10, 15, 16). It is also possible that carboxylation of ribulose diphosphate to form phosphoglycerate may be involved in succulent metabolism (2). In citrus vesicles a high activity of phospho-enol pyruvate carboxylase has been demonstrated by Huffaker and Wallace (6). Surrounded as they are by other tissues, the carbon dioxide concentration in citrus vesicles should be relatively high leading to favorable conditions for incorporation into the organic acids since this incorporation is directly dependent upon the partial pressure of carbon dioxide (1). These factors, with the data presented here, suggest the possibility that such reactions could be in part responsible for the rather large accumulation of acids in citrus fruit pulp.

## Summary and Conclusions

A comparison has been made of amount of $C^{14}O_2$ fixed in tissues of intact, small oranges and in tissue preparations of oranges in the light and the dark. Studies were made of the compounds into which the $C^{14}$-activity was incorporated.

The flavedo tissue (outer green peel) was found to be capable of rapid photosynthetic fixation of carbon dioxide. The albedo (inner white peel) and the juice vesicles showed very slight photosynthetic capacities.

In the dark, the juice vesicles were found to assimilate far more activity than the other tissues, whether in the intact fruit or as isolated tissues. Malic acid, serine, aspartic acid, and citric acid were the major labeled compounds in the orange tissues in the dark reaction.

On the basis of the high dark fixation activity of the vesicles, a proposal has been made that incorporation of $CO_2$ may have an important function in the formation and storage of acids in the citrus pulp.

## Literature Cited

1. Bonner, W. D., and J. Bonner. 1948. The role of carbon dioxide during acid formation in succulent plants. Amer. Jour. Bot. 35: 113–117.
2. Bradbeer, J. W., S. L. Ranson, and M. Stiller. 1958. Malate synthesis in Crassulacean leaves. I. The distribution of $C^{14}$ in malate of leaves exposed to $C^{14}O_2$ in the dark. Plant Physiol. 33: 66–70.
3. Calvin, M. 1951. The path of carbon in photosynthesis. The Harvey Lectures. 46: 218–251.
4. Gregory, F. G., I. Spear, and K. V. Thimann. 1954. The interrelation between $CO_2$ metabolism and photoperiodism in Kalanchoë. Plant Physiol. 29: 220–229.
5. Friedemann, T. E., and A. I. Kendall. 1929. The determination of lactic acid. Jour. Biol. Chem. 82: 23–55.
6. Huffaker, R. C. and A. Wallace. 1959. Amer. Soc. Plant Physiol., Western Section, Abstracts of Meetings.

7. KROTKOV, G., V. C. RUNECKLES, and K. V. THIMANN. 1958. Effect of light on the $CO_2$ absorption and evolution by Kalanchoë, wheat and pea leaves. Plant Physiol. 33: 289–292.
8. KUNITAKE, G., C. STITT, and P. SALTMAN. 1959. Dark fixation of $CO_2$ by tobacco leaves. Plant Physiol. 34: 123–127.
9. RACUSEN, D. W., and S. ARONOFF. 1952. Metabolism of soy bean leaves. V. The dark reactions following photosynthesis. Arch. Biochem. Biophys. 42: 25–40.
10. SALTMAN, P., G. KUNITAKE, H. SPOLTOR, and C. STITT. 1956. Dark fixation of $CO_2$ by succulent leaves. Plant Physiol. 31: 464–468.
11. TCHEN, T. T., and B. VENNESLAND. 1955. Enzymatic carbon dioxide fixation in wheat germ. Jour. Biol. Chem. 213: 533–546.
12. THOMAS, M. 1949. Physiological studies on acid metabolism in green plants. I. $CO_2$ fixation and $CO_2$ liberation in Crassulacean metabolism. New Phytol. 48: 390–420.
13. THOMAS, M. 1949. Physiological studies on acid metabolism in green plants. II. Evidence of $CO_2$ fixation in Bryophyllum and the study of diurnal fluctuation of acidity in this genus. New Phytol. 48: 421–447.
14. THOMAS, M. and H. BEEVERS. 1954. Physiological studies on acid metabolism in green plants. III. Further evidence of $CO_2$ fixation during dark acidification of plants showing Crassulacean acid metabolism. New Phytol. 53: 1–30.
15. WALKER, D. A. 1956. Malate synthesis in a cell free extract from a Crassulacean plant. Nature 178: 593–594.
16. WALKER, D. A. 1957. Physiological studies on acid metabolism. IV. Phosphoenol pyruvate carboxylase activity in extracts of Crassulacean plants. Biochem. Jour. 67: 73–79.

# CARBOHYDRATE METABOLISM OF CITRUS FRUITS I. MECHANISMS OF SUCROSE SYNTHESIS IN ORANGES AND LEMONS[1]

R. C. BEAN

DEPARTMENT OF PLANT BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, RIVERSIDE

The basis for controlling the accumulation of metabolites during growth of plants is of special interest in the study of fruit growth since the flavor and quality of the fruit may depend on the stored materials. Oranges and lemons are two fruits having wide differences in characteristic sugar storage patterns. The mature lemon contains little or no sucrose in any of its tissues whereas sucrose constitutes the major storage sugar of the orange. It would be of interest to determine if such variations are reflected in synthetic mechanisms, synthetic capacities, or in utilization of sucrose. It would also be valuable to find the stage of growth where such differences become manifest. The synthesis of sucrose in these fruits has been compared by chemical and radioisotope techniques using enzyme and tissue preparations as well as intact fruits. Results of the study suggest that differences in sucrose storage may not be due to any deficiency of sucrose synthesizing mechanisms within the lemon, since active systems were found to exist in these fruit at all stages of growth. Pronounced differences were found, however, in the synthetic capacities of different tissues of the fruit; it appears that the albedo (white peel layer) has the highest capacity for synthesis in the older fruit.

[1] Manuscript received September 22, 1959.

## MATERIALS AND METHODS

Sucrose synthesis was studied in three ways. A. Reaction of fructose or fructose-6-phosphate with uridine diphosphate glucose (UDPG) was determined in vitro with extracts or particulate preparations from the fruit. B. Formation of sucrose in isolated tissue preparations was traced by incorporation of activity from glucose-U-$C^{14}$ or fructose-U-$C^{14}$. C. The tracer technique was also extended to examine the formation of sucrose from labeled monosaccharides in small, intact fruits. Experimental procedures and materials are given below for each approach.

I. STUDIES WITH EXTRACTS AND HOMOGENATES. Mature Eureka lemon and Valencia orange fruits were obtained fresh from local groves. The lemons and oranges were juiced by hand with a glass reamer. The lemon juice was divided immediately into two parts. One part was neutralized to pH 7 with 1 *N* NaOH before carrying out subsequent steps while the remainder was treated as initially obtained. The juices were filtered through birdseye cloth and centrifuged at approximately 25,000 × G for 20 minutes. The sediments were resuspended in two volumes of 0.01 M phosphate buffer, pH 7, and dialyzed overnight against the same buffer. Supernatants from centrifuging were saturated with ammonium sulfate at pH 5 and the precipitates were collected by centrifuging. This precipitate was redissolved in the pH 7 phos-

phate buffer and dialyzed overnight in Visking sausage casing against the same buffer.

Soluble extracts of lemon and orange peels were also prepared by homogenizing one part peel in ten parts water and treating the homogenates as described above for the juices.

Sucrose synthesis was measured in the soluble preparations by modifications of previously published procedures (2, 10, 11, 12). A 50 µl aliquot of the enzyme solutions was incubated at room temperature (21–23° C) for half an hour with 0.37 µmoles of UDPG and 2.5 µmoles fructose or 2 µmoles of D-fructose-6-phosphate in a total volume of 0.1 ml. Following inactivation by heating in a boiling water bath for one minute, the residual free fructose or fructose-6-phosphate was destroyed by heating in 1 ml 0.1 *N* NaOH for 15 minutes at 100° C. The alkali-stable ketose (keto group protected by glycosidic linkage) was determined by the method of Dische and Borenfreund (8) using a 2-hour incubation rather than the specific 24-hour period. Adequate controls were prepared, omitting UDPG, enzyme or ketose.

For particulate preparations, the procedure was modified by doubling the volumes and quantities used. Following incubation, the suspension was centrifuged to remove the particulate material and an aliquot of 0.1 ml of the supernatant solution taken for analysis as above.

The UDPG was prepared from yeast extracts by a gradient elution modification of the procedure of Cabib, Leloir and Cardini (7). This material contained approximately 25 per cent of uridine diphosphate *N*-acetyl-D-glucosamine and uridine diphosphate. Potassium D-fructose-6-phosphate was obtained from the commercial barium salt by treatment with Dowex-50 (hydrogen form) followed by neutralization with potassium hydroxide.

II. Tracer Studies in Isolated Tissues. Lemons and oranges of varying ages were obtained from the groves. It was possible to obtain almost a full range of growth of the lemon fruit at a single period due to the continuous setting habit of the lemon. The range examined in oranges was limited to relatively young fruit. Each piece of fruit was cut in half and the vesicles were carefully excised. Sections of the flavedo (colored, outer layer of peel) and albedo, approximately 1.5 mm thick, were cut perpendicular to the core of the fruit. Samples in the range of 100 mg of each of these three tissues were weighed into containers and incubated at room temperature, in the dark, with 0.1 ml of a solution of labeled fructose or glucose. The solutions contained 20 µg of the active sugar with a total activity of 800,000 to 900,000 cpm per 0.1 ml aliquot. The sugars were prepared photosynthetically by the method of Putman, et al (14, 15).

Following incubation for 3 to 4 hours, the isolated tissues were inactivated and extracted with boiling ethanol. The ethanol extracts were concentrated and chromatographed by standard procedures. Water saturated phenol, butanol-acetic acid-water (52:13:35) and butanol-ethanol-water (52.5:32:15.5) solvents were used in chromatography. Active sugars were located by radioautography and the activity in each sugar determined directly on paper with a Nuclear D-47, Micro-mil end window, gas-flow counter. Sucrose was eluted from the paper and rechromatographed following hydrolysis with invertase. Activity in each of the resulting monosaccharides was determined as above.

III. Tracer Studies in Intact Fruit. Fruit in the range of 5 g total fresh weight was obtained. The stems and buttons were cut off under water and each piece of fruit was weighed quickly. A small ring of polyethylene was attached around the stem scar with the aid of a little silicone grease. The solution of carbon 14 labeled glucose or fructose was placed in the cup thus formed. An aliquot of 0.1 ml containing 1.1 mg of the sugar was used. Total activity with glucose was $9.3 \times 10^6$ cpm. For the fructose experiments $8.0 \times 10^6$ cpm was used with oranges and $16 \times 10^6$ cpm for lemons. Activity was measured with the equipment mentioned above. The fruit was allowed to take up the major portion of this solution in the dark. This required a period of about 90 minutes for the lemons and 150 minutes for the oranges. The residual solution was washed into boiling ethanol. The flavedo was rapidly cut off into hot ethanol. The fruit was then cut in half and the juice sacs excised into hot ethanol. The remaining albedo material was also inactivated in boiling ethanol after slicing it into thinner sections. After extraction with ethanol, the extracts were treated as outlined above for analysis of the isolated tissue extracts. The approximate amount of each sugar on the paper chromatograms was determined by a densitometric technique, using the color developed by reaction of the sugars with benzidine dihydrochloride (4). Density was measured with a variable intensity light source and an International Rectifier Company photocell. The signal from the photocell was amplified through a transistorized amplifier and recorded with a recording milliammeter. The amount of sugar present was determined by comparing peak heights with those peak heights found for known quantities of sugars. Using this technique, it was possible to get approximate specific activities of the sugars directly on paper.

## Results

Results for sucrose synthesis by enzyme preparations from oranges and lemons are given in table I. Data on unprocessed juices and extracts could not be obtained directly since chromogenic materials in the original preparations interfered with determination of the small amounts of sucrose formed. In the soluble extracts, this difficulty could be overcome by ammonium sulfate precipitation of the enzyme except in orange peel preparations. No difficulty was met with the resuspended particulate fractions.

TABLE I

FORMATION OF SUCROSE BY PREPARATIONS FROM LEMON AND ORANGE FRUITS

| PREPARATION | SUCROSE SYNTHESIZED | |
|---|---|---|
| | FRUCTOSE REACTION | FRUCTOSE-6-PHOSPHATE REACTION |
| | *μmoles* | *μmoles* |
| Orange Juice | | |
| Ammonium sulfate fraction | 0.12 | 0 |
| Particulate sediment | 0 | 0 |
| Lemon Juice | | |
| Ammonium sulfate fractions | | |
| pH 2.5 juice | 0 | 0 |
| pH 7 juice | 0 | 0 |
| Particulate sediment | | |
| pH 2.5 juice | 0.17 | 0 |
| pH 7 juice | 0.10 | 0 |
| Small Lemon (15 g size) homogenates | | |
| Ammonium sulfate fraction | 0 | 0 |
| Particulate sediment | 0.15 | 0 |

Conditions: 50 μl. Enzyme preparation incubated 30 minutes with 0.37 μmoles UDPG and 2.5 μmoles fructose or fructose-6-phosphate in total of 0.1 ml. Volumes and quantities doubled for analysis of particulate material and 0.1 ml of supernatant withdrawn for analysis after incubation.

The data for the juices indicate that the soluble portion of the orange juice contains an active system for the synthesis of sucrose from free fructose and UDPG. On the other hand, the lemon has no activity in the soluble fraction; instead, the particulate material shows a good capacity for sucrose synthesis. Rather than being an integral part of the particulate system, the sucrose synthesizing enzyme in lemons might be a soluble protein which is adsorbed on the particles during juicing operations due to the low pH. To test this possibility, a suspension of particles sedimented at pH 7 was divided into four portions. One part was retained for control; the other parts were divided into supernatant and sediment fractions by centrifugation. The sediments were suspended in phosphate-citrate buffer, one at pH 3.5, another at pH 5, and the third at pH 7. Each suspension was thoroughly rehomogenized in a Potter-Elvehjem apparatus, allowed to stand at 4° C for several hours with occasional shaking, and finally recentrifuged. All sediments were resuspended in phosphate buffer, pH 7, to give the original starting volume. All the final suspensions as well as the supernatant fractions were then analyzed for activity. No significant activity was found in any of the supernatants and practically all the activity (69–81 % of the original) was recovered on the particles. Thus, although this does not completely exclude the possibility that the enzyme might have been irreversibly adsorbed during processing, it tends to indicate a rather firm bond between the particles and the enzyme, in contrast to the readily soluble enzyme of the orange juice. The probability that the enzyme actually is normally associated with the particles is strengthened by the fact that it is also found in the sedimentable fraction in the preparations from the young lemons which do not have an abnormally low pH. Other enzymes, normally found in the soluble form in other organisms, have also been found in citrus associated with the particulate material (3).

Lemon tissue preparations showed an incorporation of activity into sucrose with either glucose-$C^{14}$ or fructose-$C^{14}$ at all ages of the fruit. The results of pertinent experiments are given in figure 1A, B. With glucose-$C^{14}$ as substrate no activity was found in the free fructose. Similarly, no labeling of the free glucose occurred when fructose-$C^{14}$ was used. Detectable amounts of activity were always found in the hexose phosphate although concentrations of these compounds in the citrus tissues are very low. It is evident that the albedo had a much higher capacity for sucrose synthesis per unit of fresh weight than either the juice sacs or the flavedo under these conditions. The capacity for synthesis by the albedo remained surprisingly constant throughout the fruit's growth. Activity in the flavedo and vesicles dropped off greatly in the larger fruit.

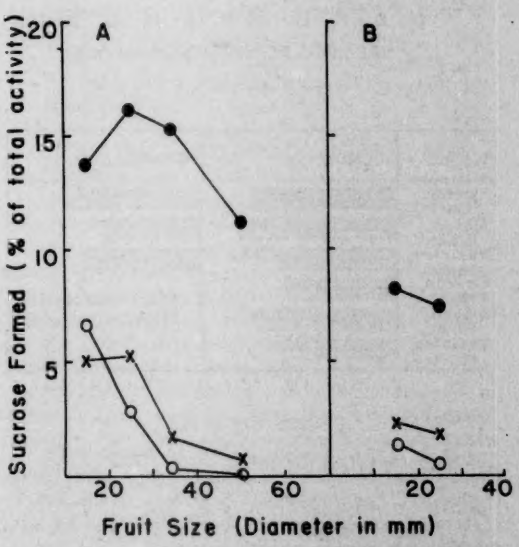
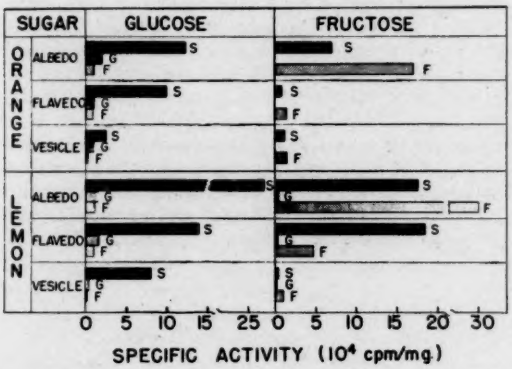



FIG. 1. A. Formation of sucrose from glucose in isolated tissues from lemons of various ages. B. Formation of sucrose from fructose in tissue preparations from lemons of two ages. ●, Sucrose formed in albedo tissue slices; ○, Sucrose formed in flavedo tissue slices; X, sucrose formed in excised vesicles. No activity was found in free fructose with glucose-$C^{14}$ as substrate nor in free glucose when fructose-$C^{14}$ was used. Total free fructose was higher than free glucose in smallest fruits approximately equal in larger fruits.

When young orange tissues were incubated with labeled glucose or fructose, no significant labeling of sucrose occurred. Instead some anomolous reactions were found (3).

Table II presents the results of the studies with intact fruits in terms of the total activity found in each sugar of the various tissues of the fruit. These data were also used to calculate the values for specific activities of the sugars given graphically in figure 2. With glucose as substrate, little activity remained in the monosaccharides; sucrose had the greatest total activity as well as the highest specific activity. On the other hand, when fructose was given, it was converted much less extensively to sucrose in both fruits. A great deal of activity remained in fructose at the end of the experimental period in all tissues. With the exception of lemon flavedo, the specific activity of fructose exceeded that of sucrose in all tissues.

Sucrose from each of the labeling experiments was subjected to hydrolysis and rechromatographed to determine the ratio of activity in glucose to activity in fructose. To determine the accuracy of the method and to furnish a control, experiments were also performed in which uniformly labeled sucrose was fed to the fruits. The resulting labeled sucrose in the fruit was hydrolyzed and measured in the same manner as the other samples. Results of this work are included in figure 3. Here it may be seen that activity was found in both components of the sucrose no matter which monosaccharide was supplied. A ratio which closely approaches unity would indicate complete equilibration of the two monosaccharides or derivatives entering into sucrose synthesis. When the sugar supplied enters into sucrose faster than it equilibrates with the other hexose precursor to sucrose a ratio greater than unity would be expected. In all these experiments an excess of the labeled monosaccharide was present through the duration of the experiment so that one might expect a higher activity in the sucrose moiety corresponding to the sugar fed unless equilibration between the two precursors to sucrose were extremely rapid. The data shows that activity was found in both components of the sucrose no matter which monosaccharide was supplied. In most cases, the monosaccharides moiety corresponding to the sugar supplied still retained a somewhat higher degree of labeling. The close approach of ratios to unity in the orange albedo indicates that a very rapid equilibration occurred between both sugars in the tissue. Lemon tissues consistently show a higher activity in the sugar which was fed than in the other sugar but even here, the activity ratios are low enough to indicate a relatively high rate of equilibration of the sucrose precursors.



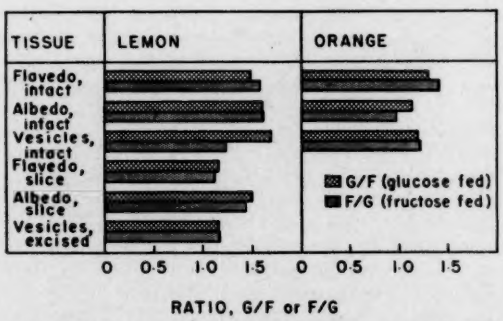




FIG. 2 (*Top*). Specific activity of sugars in the different tissues of intact lemons and oranges after uptake of glucose-$C^{14}$ or fructose-$C^{14}$, Solid bars, specific activity of sucrose; cross-hatched bars, specific activity of fructose; speckled bars, specific activity of glucose.

FIG. 3 (*Bottom*). Ratios of activities of monosaccharide components of sucrose synthesized from glucose-$C^{14}$ and fructose-$C^{14}$ in tissue preparations or intact lemon and orange fruits. Cross-hatched bars, ratio of activity of glucose to fructose from tissues given glucose-$C^{14}$, speckled bars, ratio of activity of fructose activity to glucose in tissues given fructose-$C^{14}$. Control experiments in which the ratios were determined after giving uniformly labeled sucrose indicated variability of 5 per cent in final ratios.

## DISCUSSION

Two mechanisms have been established for the synthesis of sucrose in plant materials (10, 11, 12). One mechanism involves the reaction of UDPG with free fructose to form sucrose directly while the other forms sucrose phosphate from the reaction of UDPG with fructose phosphate. Evidence presented by a number of workers (5, 6, 13) makes it appear that photosynthetic leaves use the latter reaction, exclusively. Etiolated wheat seedlings also seem to make use largely of the phosphorylated form of fructose in sucrose synthesis. Fresh, green peas have been shown to contain enzymes for synthesis of sucrose from free fructose as well as from fructose phosphate (2). In the present study it has been established that cell-free preparations from citrus fruits are capable of forming sucrose from free fructose and UDPG. No activity could be detected in these systems for the reaction of fructose-6-phosphate with

TABLE II

DISTRIBUTION OF ACTIVITY IN SUGARS FROM INTACT YOUNG LEMON AND ORANGE FRUITS*

| LABELED SUBSTRATE | SUGAR | ACTIVITY IN TISSUE/MG OF TISSUE DRY WT | | | | | |
|---|---|---|---|---|---|---|---|
| | | ORANGE FLAVEDO | ORANGE ALBEDO | ORANGE VESICLE | LEMON FLAVEDO | LEMON ALBEDO | LEMON VESICLE |
| Glucose | Glucose | 171 | 536 | 295 | 154 | 293 | 171 |
| | Fructose | 105 | 105 | 47 | 26 | 118 | 54 |
| | Sucrose | 249 | 1,420 | 1,470 | 720 | 2,810 | 3,580 |
| Fructose | Glucose | ... | ... | ... | 204 | 360 | 102 |
| | Fructose | 162 | 1,560 | 490 | 692 | 6,380 | 1,710 |
| | Sucrose | 388 | 814 | 1,150 | 4,070 | 4,420 | 1,870 |

* Total activity in each sugar per unit of dry weight.
Fruits were all in the range of 5 g total weight. Labeled sugar solutions (0.1 ml) contained 1.1 mg of sugar. Glucose activity, $9.3 \times 10^6$ cpm in both experiments. Fructose activity $8.0 \times 10^6$ cpm in the orange and $16 \times 10^6$ cpm in the lemon.

UDPG. The more indirect evidence of the tracer studies does indicate, however, that a mechanism must exist for the formation of sucrose without the utilization of free fructose and this path may be more important in the intact cell than the free fructose mechanism.

As in previous studies in intact tissues (13) the conclusion that a phosphorylated form of fructose participates in sucrose synthesis in citrus must rest mainly on the fact that the monosaccharide components within sucrose become rapidly equilibrated without similar equilibration of the free hexoses. In peel slices and intact vesicles sucrose was rapidly labeled by either glucose-$C^{14}$ or fructose-$C^{14}$. Examination of the sucrose formed showed that both hexose moieties of sucrose were labeled (fig 3). No labeling of the free fructose pool occurred with glucose-$C^{14}$ as the substrate. It is obvious from the sucrose degradation that rapid isomerization of glucose to fructose did occur before synthesis of the sucrose. Since such an isomerization is most likely to occur through the phosphorylated forms it would be safe to assume that fructose phosphate would be available for sucrose synthesis. This is supported by the fact that activity was always found in the area corresponding to the hexose phosphates. It would then seem most likely that the mechanism of synthesis of sucrose in these experiments would be phosphorylation of glucose followed by simultaneous isomerization and formation of UDPG; condensation of UDPG, and fructose phosphate could then occur with subsequent dephosphorylation of the sucrose. The observations cannot completely eliminate the possibility that free fructose might have participated in the formation of sucrose in these isolated tissue experiments but several unlikely assumptions would have to be made to support this possibility. If the free fructose is participating, the pool involved must be very small in order to account for the lack of any apparent activity in the free fructose. Since the most likely path of isomerization of glucose and fructose is through phosphorylated intermediates, an added dephosphorylation step would be required to form the active free fructose pool. This added step would tend to increase the probability of a wide spread in the activity of the two hexose moieties of the sucrose. It should also be noted that if formation of sucrose were occurring through reaction of UDPG with a small sequestered pool of free fructose the feeding of fructose-$C^{14}$ might be expected to give sucrose with a much higher activity in the fructose than the glucose moiety. This is not the case since glucose and fructose each give very similar labeling patterns in the sucrose molecule, indicating very close relationships in the pathways followed by the two sugars in entering the sucrose. Thus it would appear that the condensation of fructose phosphate with UDPG is the most likely reaction for the formation of sucrose from sugars supplied externally to isolated tissues of citrus fruits.

In the intact fruits the free fructose pool does become labeled when glucose-$C^{14}$ is the substrate so that evidence for the participation of the fructose phosphate pathway is not so unequivocal. The high specific activity of sucrose relative to fructose in the glucose utilization experiments does make it clear that the normally observed main endogenous pool of fructose cannot contribute greatly to the synthesis of sucrose. This does not exclude possible utilization of isolated small pools of free fructose which could have a high specific activity.

Results of the tissue slice experiments indicate that the albedo is the most active site of sucrose synthesis in lemons. Interfering reactions prevented similar comparisons in orange tissues. The participation of a transport system makes it difficult to assess the contribution that each tissue makes to sucrose synthesis in the intact fruit. The sugars must pass first through a vascular network in the albedo before reaching the other tissues so that many transformations could occur during the transport phase. The differences in the distribution of activity in the hexose components of sucrose isolated from the dif-

ferent tissues indicates that each tissue must make some contribution to the synthesis of sucrose. The fructose to glucose activity ratio in sucrose from the albedo of oranges fed fructose-$C^{14}$ was 1.0 (fig 3). This ratio was 1.4 in the flavedo and 1.2 in the vesicles in the same experiment indicating some dilution of the glucose portion of the sucrose may have occurred after the sugars left the albedo or that independent synthesis took place in the other tissues. Similarly, when glucose was fed, the glucose to fructose activity ratio was 1.1 in the albedo but 1.3 and 1.2, respectively, in the flavedo and juice sacs indicating changes occurring in these tissues. Intact lemons do not show the same wide differences between activity ratios in different tissues but the work with isolated tissues shows that different tissues may carry out independent synthesis of sucrose.

Axelrod and Seegmiller (1) have investigated the formation of sucrose in apple tissues and found that sucrose became labeled in both hexose moieties although not to the same extent as has been observed here.

## Summary

Formation of sucrose in oranges and lemons has been studied using tracer techniques with isolated tissues and with intact small fruit. Sucrose synthesis has also been followed by normal analytical procedures with extracts and particulate preparations.

Soluble preparations from the juice of mature oranges catalyze the reaction between uridine diphosphate glucose (UDPG) and free fructose to form sucrose. Soluble material from the juice of lemons is inactive, but particulate preparations were found to activate the same reaction. No activity could be found in these systems for catalyzing the reaction between fructose-6-phosphate and UDPG to form sucrose phosphate.

In isolated tissues (peel slices and excised vesicles) from lemons of all ages, label from glucose-$C^{14}$ or fructose-$C^{14}$ was incorporated into sucrose. Sucrose was labeled in both hexose components no matter which monosaccharide was supplied. No labeling of the free fructose occurred when glucose was given. This suggests formation of sucrose from a phosphorylated form of fructose.

Albedo tissue from lemons carried out a relatively constant rate of incorporation of glucose activity into sucrose throughout the growth of the fruit, but the rate of incorporation in the flavedo and juice sacs fell off rapidly with increase in age.

In the intact fruit, glucose is incorporated into sucrose much more rapidly than fructose. When glucose is fed, the free fructose pool also becomes labeled in both lemons and oranges. When fructose is the labeled sugar imbibed, a small amount of label appears in the free glucose of lemons but no conversion to free glucose appears in oranges.

## Literature Cited

1. Axelrod, B., and C. G. Seegmiller. 1954. Conversion of glucose to sucrose in apple tissue. Jour. Agr. Food Chem. 2: 1329.
2. Bean, R. C., and W. Z. Hassid. 1955. Synthesis of disaccharides with pea preparations. Jour. Amer. Chem. Soc. 77: 5737.
3. Bean, R. C. 1959. Glucose aerodehydrogenase from young oranges. Fed. Proc. 18: 188.
4. Bean, R. C. and G. G. Porter. 1959. Detection and differentiation of sugars and polyols on single paper chromatograms. Anal. Chem. 31: 1929.
5. Buchanan, J. G. 1953. Path of carbon in photosynthesis. XIX. The identification of sucrose phosphate in sugar beet leaves. Arch. Biochem. Biophys. 44: 140.
6. Burma, D. P. and D. C. Mortimer. 1956. The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaves. Arch. Biochem. Biophys. 62: 16.
7. Cabib, E., L. F. Leloir, and C. E. Cardini. 1953. Uridine diphosphate acetylglucosamine. Jour. Biol. Chem. 203: 1055.
8. Dische, Z., and E. Borenfreund. 1951. A new method for the detection and determination of keto sugars. Jour. Biol. Chem. 192: 583.
9. Edelman, J., V. Ginsburg, and W. Z. Hassid. 1955. Conversion of monosaccharides to sucrose and cellulose in wheat seedlings. Jour. Biol. Chem. 213: 843.
10. Leloir, L. F. and C. E. Cardini. 1953. Biosynthesis of sucrose. Jour. Amer. Chem. Soc. 75: 6084.
11. Leloir, L. F. and C. E. Cardini 1955. The biosynthesis of sucrose phosphate. Jour. Biol. Chem. 214: 157.
12. Leloir, L. F., C. E. Cardini, and J. Chiriboga. 1955. The biosynthesis of sucrose. Jour. Biol. Chem. 214: 149.
13. Putman, E. W. and W. Z. Hassid. 1954. Sugar transformations in leaves of *Canna indica*. I. Synthesis and inversion of sucrose. Jour. Biol. Chem. 207: 885.
14. Putman, E. W. and W. Z. Hassid. 1954. Isolation and purification of radioactive sugars by means of paper chromatography. Jour. Biol. Chem. 196: 749.
15. Putman, E. W., W. Z. Hassid. G. Krotkov, and H. A. Barker. 1948. Preparation of radioactive carbon-labeled sugars by photosynthesis. Jour. Biol. Chem. 173: 785.

# ABSORPTION, DISTRIBUTION, AND DESTRUCTION OF INDOLEACETIC ACID IN PLUM STEM CUTTINGS[1, 2]

D. K. STRYDOM[3] AND H. T. HARTMANN

DEPARTMENT OF POMOLOGY, UNIVERSITY OF CALIFORNIA, DAVIS

Rooting of greenwood cuttings of Marianna 2624 plum (*Prunus cerasifera* Ehrh. × *P. munsoniana* Wight & Hedr.) taken in spring or summer is dependent on the presence of a certain minimum leaf area. Treatment of the basal portion of the cuttings with indoleacetic (IAA) or indolebutyric acid (IBA) substantially increases the number of roots per cutting (20). The site of the auxin action is not known. Perhaps there is a direct effect at the site of application where roots appear. Another possibility is that auxin stimulates mobilization of root-promoting substances from the leaves; these substances would then presumably be translocated basipetally to the site of root initiation.

Although much study has been given to the absorption and translocation of auxins in intact plants, very few workers have investigated these phenomena in detached parts of woody plants. Cooper (4), using the standard Avena curvature test to follow movement of IAA in the bark of leafless lemon cuttings found initially relatively large amounts of IAA in the region of treatment; however, 95 per cent of the auxin disappeared during the 1st day, and little auxin was translocated to the apices of the cuttings. Turetskaya (24) followed the distribution of $I^{131}$ in soft and hardwood cuttings of various species treated with $I^{131}$-labeled-*p*-iodophenoxyacetic acid. In the hardwood cuttings the maximum $I^{131}$ accumulation occurred in the base of the stem where the roots were formed. Decreasing amounts of $I^{131}$ were found in xylem, bark, and buds, in that order. Turetskaya (25) followed the movement of $C^{14}$-carboxyl-labeled naphthaleneacetic acid in leafy cuttings of balsam, cherry, and currant, and found some evidence that the NAA was metabolized.

The present study was designed to follow the absorption, translocation, and persistence of $C^{14}$ from alpha-labeled IAA in cuttings of Marianna 2624 plum.

## MATERIALS AND METHODS

Leafy, stem cuttings of Marianna 2624 plum were taken at various times from June to November as follows: Sub-terminal cuttings of current season's growth were prepared from 2-year-old nursery trees and 9-year-old orchard trees. These were made by severing the distal end of the shoot at the site of the first fully expanded leaf, followed by a second cut about eight inches down the shoot.

About two hours elapsed between cutting preparation and experimentation. Four types of samplings were made from the original cuttings: A. Entire cuttings 5½ to 8 in long; B. Stem pieces ¼ to ¾ in long; C. Leachings prepared by soaking stem pieces; D. A brei prepared by blending 80 g of stem tissue in 50 ml of water for 10 minutes in a Waring blendor. Intact cuttings were used to study absorption and destruction of indoleacetic acid. Stem pieces, leachings, and breis were used for auxin destruction studies only.

Unless noted otherwise, cuttings were divided into "leafless" and "leafy" classes. All leaves were removed from the former while a single apical leaf was left in the latter. When auxin-dip treatments were made, the basal 1½ in of the cutting was removed just before treatment to provide a freshly-cut surface for treatment. When treatments were made at the stem apex, ½ in of stem was left above the leaf to be treated with the labeled solution.

Indoleacetic acid labeled in the alpha carbon with $C^{14}$ was obtained from Tracerlab, Inc., of Waltham, Mass. The 15.9 mgs secured contained 0.1 millicurie $C^{14}$ (specific activity 1.1 millicuries per millimole). This material, plus enough unlabeled IAA to make a 4,000 ppm solution of the auxin, was dissolved in 50 ml of 50 % ethyl alcohol.

Cuttings were given auxin treatment by dipping the apical ½ in or the basal 1½ in into the IAA solution for 5 (or sometimes 10) seconds. The solution adhering to the surface of the cutting was allowed to evaporate; this usually required about five minutes. Cuttings to be used in short-term experiments were wrapped in moist filter paper. Cuttings for long-term experiments were placed in an intermittent mist propagating bed in the greenhouse (9).

Two experiments were conducted using the radioautographic method of Yamaguchi and Crafts (29) to follow the translocation of the labeled auxin in the cuttings. The first consisted of basal treatments only. Samples were taken at the following time intervals after treatment: 10 minutes; 1 hour, 1½ hours, 24 hours; 2, 4, 7, 12, and 28 days. The second experiment included apical as well as basal treatments. Samples from apically-treated cuttings were taken after 5 hours, 4 days, and 20 days. Samples from basally-treated cuttings were taken after 1½ hours, 4 days, and 20 days. In each case, the cuttings were divided into 1 in segments. Cross and median longitudinal sections were prepared, freeze-dried and radioautographed. One untreated cutting was used to check for pseudo-autography by volatile emanations from the tissues.

---

[1] Received revised manuscript September 14, 1959.

[2] The assistance of Dr. E. C. Maxie and Miss Betty Robinson throughout this study is gratefully acknowledged.

[3] Present address: Western Province Fruit Research Station, Stellenbosch, Union of South Africa.

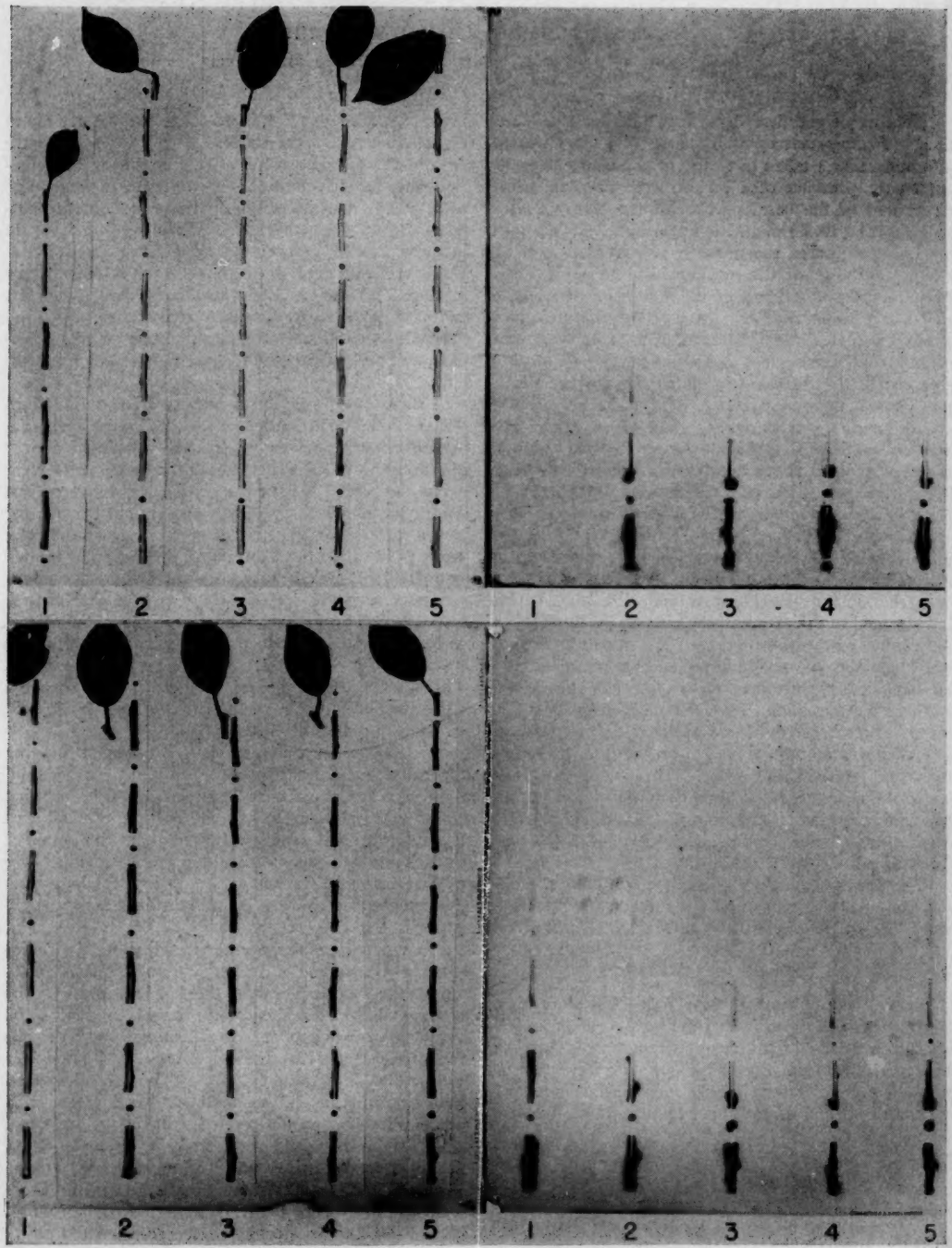



FIG. 1. Distribution of radioactivity in Marianna 2624 plum stem cuttings treated by immersing the basal 1¼ in for 5 seconds in a 4,000 ppm alpha-carbon labeled indoleacetic acid solution.

(*Top, left*). Original mounts. (*Top, right*). Radioautographs. (*left to right*): 1). No treatment, 2). 10 minutes, 3). 1 hour, 4). 1½ hours, and 5). 24 hours after treatment.

(*Bottom, left*). Original mounts. (*Bottom, right*). Radioautographs. (*left to right*): 1). 2 days, 2). 2 days, 3). 4 days, 4). 7 days, 5). 12 days after treatment.

Two experiments using $C^{14}$ counting techniques were conducted to study the absorption, translocation, and destruction of labeled IAA. In the first experiment 50 cuttings were used. In contrast to the leafy cuttings described above, only the leaves from the basal 2 in of the cuttings were removed. After treatment with labeled IAA, this sample was divided into five groups, and assayed for $C^{14}$ activity after 0, 1, 2, 5, and 12 days. The second experiment was designed to obtain an approximation of the amount of IAA absorbed per cutting. A total of five leafy and four leafless cuttings was used in this experiment. Following treatment, the treated surfaces of the cuttings were carefully washed with 70 % alcohol. The samples were prepared for counting as described below. In both of these experiments, the cuttings were 6 in long. Each cutting was divided into three 2 in pieces. All the corresponding segments were combined as single samples, dried to a constant weight in a vacuum oven at 68° F, and ground to pass a 20-mesh screen in a Wiley mill. Total carbon and carbon 14 activity were measured as described by Maxie et al (12), except that the counting was done by a Tracerlab TGC-14 thin window tube, mounted in an automatic sample changer. The total number of counts for each determination was 600, and four determinations were made for each sample. Background and standard $BaC^{14}O_3$ plates were counted in the same way. Student's t-test was used in the statistical analysis of the data.

The $C^{14}$ activity in respiratory carbon dioxide was measured by sealing treated cuttings in a quart jar covered by black cloth. Carbon dioxide-free air was passed over the cuttings, and the respiratory $CO_2$ trapped, plated, and counted as described above.

Destruction of IAA by tissue leachings and breis was followed by the colorimetric method of Gordon and Weber (8), using an Evelyn colorimeter with a 520 filter. Leachings were prepared by cutting stems into ¼ in pieces. Eight grams of such pieces were placed in each of two beakers. One lot was autoclaved for 1 hour at a pressure of 15 pounds per square inch, after which the stem pieces were transferred to beakers containing 10 ml of a 100 ppm solution of $C^{14}$-labeled IAA. The beakers were placed under a black cloth and kept at room temperature. One milliliter aliquots were removed at the various time intervals for analysis. Breis were tested by using 1 ml aliquots from the filtered solutions.

Peroxidase activities of leachings were measured by the oxidation of pyrogallol by hydrogen peroxide. To 1 ml of leaching was added 2 ml of 1 % pyrogallol, 3.8 ml distilled water, and 0.2 ml of 3 % hydrogen peroxide. Color changes were followed with the Evelyn colorimeter using a 425 filter.

For the manometric assay of IAA oxidation, the brei described earlier was centrifuged 10 minutes at 2000 × G. The supernatant was decanted from the cellular debris and used as the enzyme source. Oxygen uptake was measured by the direct method described by Umbreit et al (26) at 25° C. The main reaction chamber contained 1.5 ml of the enzyme preparation, 0.5 ml of 0.1 M succinate buffer pH 5.0; 0.3 ml of 0.01 M manganese chloride; and 0.2 ml of 0.01 M resorcinol with 0.15 ml 20 % potassium hydroxide in the center well. The sidearm contained 0.5 ml of 0.01 M IAA. The flasks were shaken in the water bath for 25 minutes, the sidearm contents tipped in, and readings made for a period of 3 hours.

To test for IAA destruction within fresh and autoclaved tissues, 15 stem pieces ¾ in long were taken from each treatment. These were immersed for 10 seconds in a solution of $C^{14}$-labeled IAA, dried, and placed in two 10 ml beakers. Each beaker was affixed with modeling clay to the inner wall near the top of a quart jar. Twenty milliliters of 1 M sodium hydroxide was pipetted into the bottom of the jar, and the system sealed. The jars were covered with black cloth. After 60 hours at room temperature, the carbonate in the base was precipitated and assayed for $C^{14}$ activity as noted above.

## Results

The results of the radioautographic experiments are shown in figures 1 and 2. Figure 1 (top) shows the distribution of $C^{14}$ after 10 minutes, 1, 1½, and 24 hours. A very rapid movement of the $C^{14}$ is apparent. Close observation of the autograph shows radioactivity in the fourth 1 inch segment of the cuttings after all four time intervals. The epidermis of the treated portion of the stem showed very high $C^{14}$ activity, as would be expected. Initially, most of the activity was in the new xylem. None of the cuttings showed radioactivity in the leaves, and no pseudoautography was observed.

Figure 1 (bottom) shows the distribution of the radioactivity at 2, 4, 7, and 12 days after treatment. At the end of this period most of the activity was still confined to the basal 4 in of the cuttings. Lateral movement from the xylem into the phloem had occurred, and in the original autographs faint images of $C^{14}$ activity could be detected in the leaves at all collection dates. The cutting collected on the 12th day showed extensive callus development in the region of IAA treatment.

Figure 2 (top) illustrates a comparison of the distribution of $C^{14}$ activity in leafy and leafless cuttings. In the case of the basally treated leafless cuttings, the activity was detectable in the 4th inch after 1½ hours and in the 5th inch after 4 days.

In the apically treated leafy cuttings, the $C^{14}$ had moved downward to the 5th inch in detectable amounts after 5 hours and persisted there for 4 days after treatment. Negligible amounts of radioactivity were present in the leaves immediately adjacent to the treated portion.

Roots had developed after 28 days on the basally-treated leafy cutting as shown in figure 2 (bottom). Autographically demonstrable $C^{14}$ activity could be observed in the roots, stem, and leaf. In the apically-treated and leafless cuttings, radioactivity was detectable in all parts of the cutting 20 days after treatment but no callus or roots developed at either end

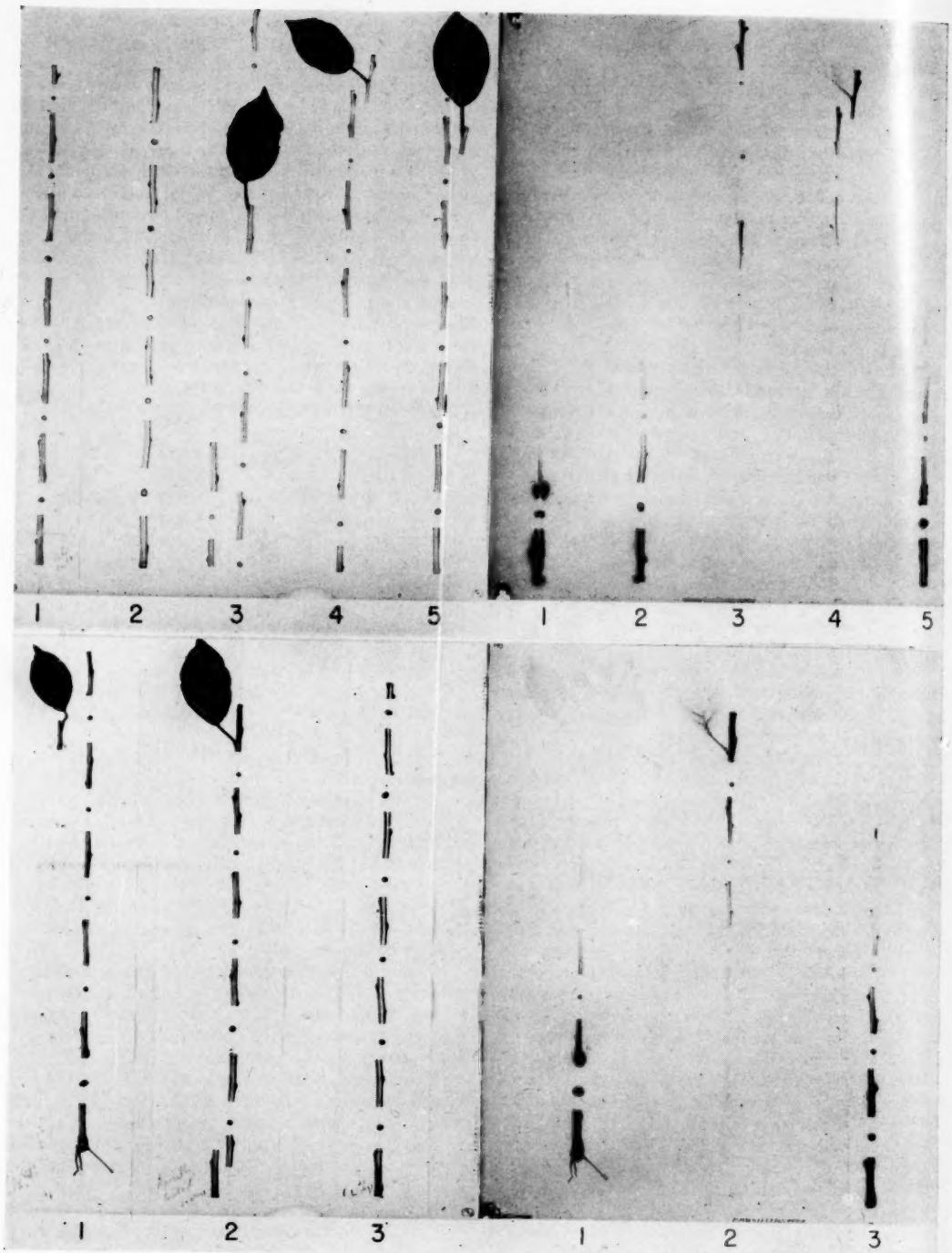



FIG. 2. Distribution of radioactivity in Marianna 2624 plum stem cuttings treated by immersing the basal 1¼ in for 5 seconds in a 4,000 ppm alpha-carbon labeled indoleacetic acid solution.

(*Top, left*). Original mounts. (*Top, right*). Radioautographs. (*left to right*): 1). Leafless cutting, basally-treated, 1½ hours after treatment. 2). Leafless cutting, basally-treated, 4 days after treatment. 3). Single-leaved

TABLE I

DISTRIBUTION OF $C^{14}$ FROM ALPHA-LABELED INDOLEACETIC ACID IN MARIANNA 2624 PLUM CUTTINGS*

| DAYS AFTER TREATMENT (10 SEC DIP OF BASAL 1½ IN OF CUTTINGS IN α-LABELED IAA) | POSITION ON CUTTING | | | | | | | | | | TOTAL μC/G D. WT, SAMPLE × $10^{-4}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | BASAL THIRD | | | | MIDDLE THIRD | | | | APICAL THIRD | | |
| | STEM | | LEAVES | | STEM | | LEAVES | | STEM | | |
| | COUNTS/ MIN | μC/G D. WT, × $10^{-4}$ | COUNTS/ MIN | μC/G D. WT, × $10^{-4}$ | COUNTS/ MIN | μC/G D. WT, × $10^{-4}$ | COUNTS/ MIN | μC/G D. WT, × $10^{-4}$ | COUNTS/ MIN | μC/G D. WT, × $10^{-4}$ | |
| 0 | 72.91 | 163.83 | 0 | 0 | 1.96** | 5.74 | 0 | 0 | 0 | 0 | 34.7 |
| 1 | 39.41 | 80.68 | 3.03 | 6.90 | 10.84 | 22.24 | 2.20 | 4.22 | 2.62 | 5.42 | 24.0 |
| 5 | 47.50 | 83.24 | 1.02NS | 3.40 | 8.60 | 17.07 | 1.18NS | 2.66 | 2.09 | 4.60 | 20.8 |
| 12 | 27.16 | 55.36 | 1.89** | 3.24 | 8.74 | 17.01 | 2.07** | 2.80 | 0.94NS | 0.98 | 17.7 |

* Statistical treatment applied to counts per minute only.
NS—not significant at 5 per cent level.
** Significant at 5 per cent level. (All others significant at 1 % level.)

after 28 days. Similarly, basally-treated leafless cuttings showed no callus or root development in this time.

As corroboration of the radioautographs, the distribution of $C^{14}$ in treated cuttings was determined by counting techniques. The data, as given in table I, show a decrease in the total radioactivity in the individual samples with time. The most rapid decrease occurred within 24 hours after treatment, when the activity dropped from 34.7 × $10^{-4}$ microcuries per gram dry weight to 24.0 × $10^{-4}$ microcuries. Immediately after treatment a low, but significant, amount (5.74 × $10^{-4}$ microcuries per gram dry weight) of radioactivity was present in the stem section adjacent to the treated basal third but the leaves showed no $C^{14}$ activity. After 24 hours, low but significant amounts of radioactivity were found in all parts of the cutting. Thereafter, the radioactivity in the upper 4 in of the cutting was relatively constant despite a consistent decline in the cutting as a whole. After 12 days, the cuttings still contained approximately 50 % of the $C^{14}$ activity noted immediately after treatment.

The radioautographs showed that leafless and leafy cuttings apparently absorbed equal amounts of the alcoholic IAA solution. This observation was confirmed by the counting technique. Each leafless cutting had 12.4 × $10^{-4}$ microcuries, while each leafy cutting had 11.8 × $10^{-4}$ microcuries. From the specific activity it was calculated that each cutting had absorbed 0.0023 mg of IAA.

The results of the experiment to determine whether $C^{14}O_2$ was evolved by cuttings treated with labeled IAA are shown in figure 3. During the first 12 hours after treatment, $C^{14}O_2$ was evolved at the average rate of 3.04 × $10^{-6}$ microcuries per hour (2 net counts/min). Sixty hours after treatment, radioactive $CO_2$ was being evolved at the rate of 5.0 × $10^{-4}$ microcuries per hour (675 counts/min). Following this peak, there was a rapid decrease in the radioactivity of the respiratory $CO_2$ until the 7th day. Thereafter, the $C^{14}$ activity was relatively constant until the experiment was terminated. When tissue slices were treated with labeled IAA, the respiratory $CO_2$ showed 54.1, 63.1, and 98.4 counts per minute after 1, 2, and 4 hours, respectively. To determine if the auxin destruction was enzymatic, both intact and autoclaved stem pieces were dipped in the labeled IAA. Carbon dioxide was trapped for 60 hours and assayed for total carbon and $C^{14}$ activity. The intact tissues yielded carbonate having 15.7 net counts per minute (6.7 × $10^{-4}$ microcuries of $C^{14}$). The autoclaved samples yielded insignificant amounts of carbonate and contained no measurable radioactivity.

When the leachings from cuttings were checked for indoleacetic acid oxidase activity with the Salkowski reagent, an indication of a rapid destruction of IAA was obtained. To ascertain whether this ap-

cutting, apically-treated, 5 hours after treatment. 4). Single-leaved cutting, apically-treated, 4 days after treatment. 5). Single-leaved cutting, basally-treated, 4 days after treatment.

(*Bottom, left*). Original mounts. (*Bottom, right*). Radioautographs. (*left to right*): 1). Single-leaved cutting, basally-treated, 28 days after treatment. 2). Single-leaved cutting, apically-treated, 20 days after treatment. 3). Leafless cutting, basally-treated, 20 days after treatment.

parent destruction was an enzymatic phenomenon or an artifact, leachings from autoclaved tissues were also tested. The results showed that autoclaved leachings apparently destroyed IAA at 1.5 times the rate of non-autoclaved preparations—0.138 and 0.086 micromoles destroyed in 2 hours, respectively. When two preparations obtained from breis of intact and autoclaved tissues, respectively, were assayed for IAA oxidase activity with the Warburg apparatus, no oxygen uptake was observed in either case. The same reaction mixture when tested by the Salkowski reaction showed a marked apparent destruction of IAA. Using the same concentration of IAA, it was found that adding small amounts of catechol in the absence of plant extract gave results quite similar to that noted with autoclaved leachings.

The results of an assay for peroxidase activity in the leachings from autoclaved and non-autoclaved tissues showed that non-autoclaved tissues had a very active peroxidase and the autoclaved tissues none. There was no regeneration of the peroxidase in the autoclaved tissues after 24 or more hours.

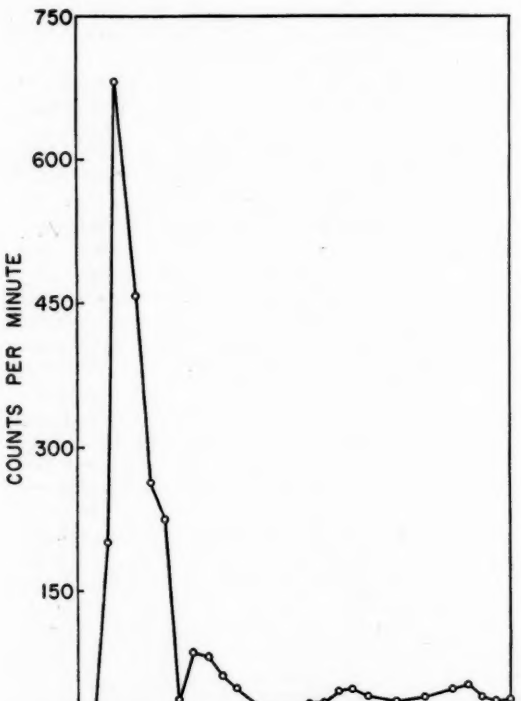



FIG. 3. Radioactivity in the respiratory $CO_2$ of Marianna 2624 plum stem cuttings treated with a 4,000 ppm alpha-carbon labeled indoleacetic acid solution for 10 seconds.

## DISCUSSION

Good agreement was obtained between the radioautographic and counting methods as a measure of the absorption and translocation of labeled IAA. Both techniques showed a rapid absorption and movement of IAA. Most of the radioactivity remained in the basal portion of the cuttings with only slight amounts distributed through the rest of the cuttings. This situation persisted from 1 day after treatment until rooting occurred.

It seems that some mechanism other than transpirational pull must have been responsible for the rapid uptake of IAA in the cuttings, since leafless cuttings absorbed the same amount of IAA as did leafy cuttings.

Since the $C^{14}$ assay gives no indication as to the nature of the compounds in which the $C^{14}$ activity is located it was not possible to compare our results with those of Cooper (4), who used the standard Avena curvature test to determine the auxin distribution in basally-treated cuttings. Similarly, it is not possible to compare these results with those of Turetskaya (25), who dipped cuttings into relatively weak NAA solutions for 15 hours.

Whether the early distribution of radioactivity through the cutting was important in root initiation is not known since the identity of the compound(s) containing the radioactivity was not determined. This is especially true when one considers the work of Andreae et al (1, 7), and Fang and his co-workers (5), who found that applied IAA conjugated with aspartic acid and ammonia and very little free IAA accumulated. Some of these conjugates had auxin activity varying from very weak to that comparable to IAA, depending on the assay method used.

It is doubtful that the radioactivity found in the roots of the cuttings was still in the IAA molecule since it is well known that roots have a strong ability to destroy IAA (28). In addition, Bonner and Bonner (2) believe that an essential feature of root induction by auxins is that the active substance must have disappeared before emergence of the new roots.

From the data obtained in this study, it is not possible to determine if IAA changed the rooting behavior of the cuttings by mobilization of some factor(s) in the leaf.

From the counting data, it was evident that the total amount of radioactivity in the cuttings decreased with time. The sharp decrease that occurred within the 1st day after treatment probably is not due to enzymatic destruction of IAA. Since in this experiment no effort was made to remove the IAA solution from the surface of the basal ends of the cuttings after treatment, and since the cuttings were placed under intermittent mist, much of the loss in the first 24 hours was probably due to washing the labeled solution from the surface. However, it seems likely that most of the subsequent disappearance of radioactivity could be ascribed to enzymatic destruction. This is supported by the persistence of $C^{14}$ in the respiratory carbon dioxide for more than 4 weeks after treatment. As

shown in figure 3 a very marked destruction of IAA occurred after an initial lag period of 1 day. This lag period could represent the time required for the IAA to move from the xylem, where it first accumulated, to the phloem, where it came in contact with living cells, capable of metabolizing the auxin. This explanation seems probable since measurements of the radioactivity of respiratory $CO_2$ showed that tissue sections treated with labeled IAA for 10 seconds could metabolize IAA within 1 hour after treatment. Presumably the lag period noted with whole cuttings represented the time it took for complete distribution of the labeled auxin throughout the cutting

Although much work has been done to determine the mechanism whereby IAA is oxidized by IAA oxidase, the results have been conflicting. The same situation exists regarding the identity of the products of IAA metabolism (11, 16, 17, 19, 22, 23, 27). According to Ray (18), the lack of agreement as to the products of IAA oxidation is due to the fact that an unstable intermediate is formed which subsequently yields two or more substances.

To study the products of IAA oxidation, much use has been made of radioactive IAA. Radioactive $CO_2$ has been found in all cases where carboxyl-labeled IAA was used, either with purified enzyme systems or with tissue sections. In the one instance where alpha-labeled IAA was employed, no radioactive $CO_2$ was observed (22), but the purified enzyme used in this case was able to form a number of radioactive products.

As to the nature of the IAA oxidizing enzyme, Ray (18) stated that most of the IAA oxidation in homogenates can be accounted for by peroxidases. Bonner (3) stated that there is no one specific IAA oxidase, but rather that a variety of IAA oxidizing systems seem to be produced as a result of cell rupture. In this study it was found that the alpha carbon appeared in the respiratory $CO_2$ in less than 1 hour from the time of treatment when tissue sections were used. Two explanations seem possible to account for this, and both possibilities require a cleavage of the indole ring as occurs in tryptophane degradation (13). Once the ring is broken a $\beta$-oxidation could occur as it does in the case of fatty acid oxidation, or alternatively, two successive alpha-oxidations could occur as in the case of the oxidation of long chain fatty acids by fatty acid peroxidase (21).

The results of this study imply that the mechanism for IAA metabolism by tissue slices may be different from that operating when purified enzyme systems are used.

It is well known that $\beta$-oxidation involves the formation of acetyl coenzyme (Co.A). Once this is formed, the labeled carbon can appear in a wide variety of compounds such as respiratory $CO_2$, organic acids, amino acids, and proteins. Any of these could account for the appearance of radioactivity in the respiratory $CO_2$ and in the newly formed roots.

No oxygen uptake by preparations obtained from breis of non-autoclaved tissue was observed. This could be ascribed to the fact that no attempt was made to control the pH and tonicity during blending of the tissue. In addition, naturally-occurring inhibitors could have been present.

Our experiments suggested two possible mechanisms for auxin destruction in cuttings of Marianna 2624 plum. One was clearly enzymatic as evidenced by evolution of $C^{14}O_2$ in non-autoclaved tissues. The other was apparently non-enzymatic as shown by autoclaved tissues when tested by the Salkowski reagent. When the latter was pursued further, however, it became clear that an artifact was involved in the Salkowski test. This was evident for three reasons. First, the autoclaved tissues were more effective in the apparent destruction of IAA than were the intact tissues; if the apparent destruction was enzymatic, a decrease in activity, if not complete inactivation, would have been expected. Second, the autoclaved tissues did not show peroxidase activity. The degree of heat inactivation of this enzyme often is used as a measure of completeness of enzyme destruction in canned foods; it seems very improbable that an IAA oxidase would be more heat stable than peroxidase. Finally, no $C^{14}$ liberation occurred when the labeled auxin was incubated with autoclaved tissues.

Platt and Thimann (15) have warned against the use of the Salkowski test with plants having a high level of phenols. The cut surfaces of the Marianna 2624 plum stem were noted to darken quite readily, indicating phenolase activity on endogenous phenols. The tissues were shown to have phenolase activity by their capacity to oxidize added catechol as measured by Warburg manometry. Therefore, it appeared that these tissues were capable of interference with the Salkowski test noted by Platt and Thimann (15). Indeed, addition of small amounts of catechol to IAA in the absence of enzymes gave a Salkowski color test analagous to that obtained with leachings from autoclaved tissues. From these results it is clear that the apparent destruction of IAA as measured by the Salkowski test was an artifact. The greater capacity of autoclaved tissues to destroy IAA probably reflects an increased release of phenolic substances from the tissues by heat destruction of cytoplasmic membranes. In this regard, the results reported by Phipps (14) are open to question, for she reported $O_2$-independent auxin destruction by leaf extracts of potato and tomato which was increased by heating. She used the Salkowski test, and was perhaps observing phenolic interference with the test, similar to that reported here.

## Summary

I. The absorption, translocation, distribution, and destruction of alpha-labeled IAA was determined in Marianna 2624 plum stem cuttings, using radioautographic and counting techniques.

II. No difference was observed in the amount of IAA taken up by leafy and leafless cuttings treated by the concentrated-solution-dip method.

III. At the concentration used in these experiments, the IAA moved in both acropetal and basipetal directions. In leafy cuttings the radioactivity was distributed throughout the entire cutting 24 hours after treatment.

IV. Adventitious roots induced by IAA treatment showed appreciable amounts of radioactivity 28 days after the cuttings were treated. At this time considerable radioactivity was still evident in all parts of the cutting.

V. Cuttings treated with alpha-labeled IAA showed detectable amounts of radioactivity in the respiratory $CO_2$ within 12 hours from treatment. Within 60 hours the respiratory $CO_2$, precipitated as $BaC^{14}O_3$ counted 675 net counts per minute. By the 7th day this value had decreased to 42 net counts per minute. The respiratory $CO_2$ still showed some radioactivity 30 days after treatment.

VI. Tissue slices prepared from Marianna 2624 plum stem tissue metabolized alpha-labeled IAA very readily since appreciable amounts of $C^{14}O_2$ were detected within 1 hour after treatment.

VII. Homogenates prepared from both intact and autoclaved plum stem tissue showed an apparent destruction of IAA as measured by the Salkowski reaction. Peroxidase activity could only be demonstrated in the homogenate obtained from the intact tissue. The data obtained suggested that the destruction was due to an interference of endogenous phenolic substances with the Salkowski reaction.

VIII. Two possible mechanisms are suggested for the oxidation of IAA by the plum stem tissue.

## Literature Cited


1. Andreae, W. A., and N. E. Good. 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382.
2. Bonner, J., and H. Bonner. 1948. The B vitamins as plant hormones. Vitamins and Hormones 6: 225–275.
3. Bonner, W. D. 1957. Soluble oxidases and their functions. Ann. Rev. Plant Physiol. 8: 427–452.
4. Cooper, W. C. 1938. Hormones and root formation. Bot. Gaz. 99: 599–614.
5. Fang, S. C., P. Theisen, and J. S. Butts. 1959. Metabolic studies of applied indoleacetic acid-1-$C^{14}$ in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34: 26–32.
6. Galston, A. W., and L. Y. Dalberg. 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. Amer. Jour. Bot. 41: 373–380.
7. Good, N. E., W. A. Andreae, and M. W. H. van Ysselstein. 1956. Studies on 3-indoleacetic acid metabolism. II. Some products of the metabolism of exogenous indoleacetic acid in plant tissue. Plant Physiol. 31: 231–235.
8. Gordon, S. A., and R. P. Weber. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192–195.
9. Hartmann, H. T. and D. E. Kester. 1959. Plant Propagation: Principles and Practices. Prentice-Hall, Englewood Cliffs, N. J.
10. Maclachlan, G. A., and E. R. Waygood. 1956. Kinetics of the enzymatically-catalyzed oxidation of indoleacetic acid. Can. Jour. Biochem. Physiol. 34: 1233–1250.
11. Manning, D. T., and A. W. Galston. 1955. On the nature of the enzymatically catalyzed oxidation products of indoleacetic acid. Plant Physiol. 30: 225–231.
12. Maxie, E. C., M. V. Bradley, B. J. Robinson, and A. A. Hewitt. 1959. Distribution of $C^{14}$ from carboxyl-labeled 2,4,5-trichlorophenoxyacetic acid in fruit tissues of Tilton apricot. Proc. Amer. Soc. Hort. Sci. 73: 134–144.
13. Meister, A. 1957. Biochemistry of the Amino Acids. Academic Press, New York.
14. Phipps, J. 1958. Sur la destruction in vitro de l'acide indoleacetique par les extraits de feuilles de Tabac on de Pomme de terre. Compt. rend. 246: 3269–3271.
15. Platt, R. S., and K. V. Thimann. 1956. Interference in Salkowski assay of indoleacetic acid. Science 123: 105–106.
16. Proctor, M. H. 1958. Bacterial dissimilation of indoleacetic: A new route of breakdown of the indole nucleus. Nature 181: 1345.
17. Racusen, D. 1955. Formation of indole-3-aldehyde by indoleacetic acid oxidase. Arch. Biochem. Biophys. 58: 508–509.
18. Ray, P. M. 1958. Destruction of auxin. Ann. Rev. Plant Physiol. 9: 81–118.
19. Ray, P. M., and K. V. Thimann. 1955. Steps in the oxidation of indoleacetic acid. Science 122: 187–188.
20. Strydom, D. K. 1959. Physiological and biochemical aspects of root initiation in detached woody stem tissue. Ph.D. dissertation, University of California, Davis.
21. Stumpf, P. K., and C. Bradbeer. 1959. Fat metabolism in higher plants. Ann. Rev. Plant Physiol. 10: 197–222.
22. Stutz, R. E. 1958. Enzymatic formation of indole-3-carboxaldehyde from indole-3-acetic acid. Plant Physiol. 33: 207–212.
23. Tang, Y. U., and J. Bonner. 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25.
24. Turetskaya, R. Kh. 1954. The absorption and distribution of radioactive iodine used in a growth stimulator on cuttings. Izvest. Akad. Nauk. S.S.S.R. Ser. Biol. 4: 35–41.
25. Turetskaya, R, Kh. 1957. Radioactive carbon investigation of the uptake and distribution of growth regulators in plant cuttings. Plant Physiol. (Fiziologiya Rastenay) 4(1): 42–51.
26. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1951. Manometric Techniques and Tissue Metabolism. Burgess, Minneapolis.
27. Wagenknecht, A. C., and R. H. Burris. 1950. Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. Arch. Biochem. 25: 30–53.
28. Went, F. W., and K. V. Thimann. 1937. Phytohormones. MacMillan, New York.
29. Yamaguchi, S., and A. S. Crafts. 1958. Autoradiographic method for studying absorption and translocation of herbicides using $C^{14}$-labeled compounds. Hilgardia 28: 161–191.

# EFFECT OF WATER STRESS ON CELL WALL METABOLISM OF AVENA COLEOPTILE TISSUE [1]

LAWRENCE ORDIN [2]

INSTITUTE OF SOIL SCIENCE, AGRICULTURAL RESEARCH STATION, REHOVOT, ISRAEL

Many papers published during the past two decades have shown the close relationship between moisture stress and growth. Moisture stress may be defined as the diffusion pressure deficit (DPD) of the water in the medium supporting a plant or surrounding isolated plant parts. It can consist in whole or in part of osmotic pressure (OP) due to dissolved solutes in the water. Reviews of the literature are given by Crafts, Currier, and Stocking (6), Richards and Wadleigh (15), and more recently by Slatyer (18). Wadleigh and Gauch (20) showed how rate of leaf elongation in cotton is closely dependent upon the soil moisture stress. In the Avena coleoptile the growth variable most sensitive to various factors is elongation which is accompanied by an increase in cell wall material at room temperatures, but not at 2° C. as shown by Bonner (2). Elongation in this tissue is reduced markedly by an increase in moisture stress in the surrounding medium (11).

Since some aspect of cell wall metabolism appears to be directly involved in cell elongation (4), it is particularly interesting to investigate the cell wall with respect to water stress. In the plant cell the DPD consists of the internal OP minus the wall pressure which equals turgor pressure (TP). In the case of the coleoptile section the cell DPD equals the medium DPD (11).

In a previous paper (10) it was shown that an increase in DPD of coleoptile tissue reduces the amount of $C^{14}$ incorporated from glucose-$C^{14}$ into various cell wall constituents. There was a preliminary suggestion that by increasing TP, salt absorption results in increased $C^{14}$ incorporation into cell wall constituents. It was not clear, however, if all of the effects observed could be ascribed only to an increase in TP and not also to an increase in internal OP.

This investigation was undertaken to evaluate more accurately the effects on cell wall metabolism of factors influencing the DPD and TP of the tissue.

## MATERIALS AND METHODS

In this investigation variation of mannitol concentration in the medium and exposure to salt was used to vary DPD and internal OP (leading to variations in TP).

Avena seedlings (variety Rodney) were grown for 96 hours in enameled trays containing vermiculite. These were maintained at 26° C under red-orange light and were watered daily with deionized water. Harvesting of sections and all subsequent operations were carried out in diffuse daylight except pretreatments and growth tests, which were carried out in the dark. Coleoptiles of 25 to 35 mm length were selected. Primary leaves were removed and 5 mm sections cut 2 to 3 mm from the tip of each coleoptile were used.

Various pretreatments which will be described later were carried out in 20 ml of solution in Petri dishes. Groups of 100 sections were then floated for 3 hours in 3.5 ml each of treatment solutions containing glucose-$C^{14}$ in 50 ml Pyrex beakers which were slowly shaken at a rate of about 50 strokes per minute. The radioactive solutions contained 1 microcurie per vessel of uniformly labeled glucose-$C^{14}$ undiluted by added carrier. The glucose which had a specific activity of 67.5 microcuries per mg glucose was purchased from the Radiochemical Centre, Amersham, England. Temperatures on the shaker are given in the accompanying tables. At the end of the incubation period the sections were transferred to a fritted glass plate filter funnel and rinsed several times with water accompanied by suction. The tissue was then frozen in liquid air and stored frozen until analyzed. A supplementary experiment showed that this method of rinsing gave the same value of total $C^{14}$ uptake by intact sections as the uptake obtained either with coleoptile sections from which leaves have not been removed or with leafless sections which were blotted dry by pressure (sufficient to expel water from the interior hollow cylinders) on filter paper, and which were then rinsed with water and reblotted.

The frozen tissue was ground in cold water in a glass mortar; the homogenate then was transferred to a fritted glass plate filter funnel. The stem of this funnel was bent in an inverted U shape to form a Weihe-Phillips extractor similar to that described by Kertesz (8). The homogenate was washed

[1] Received September 15, 1959.

[2] Present address: Department of Plant Biochemistry, Citrus Experiment Station, University of California, Riverside.

thoroughly with cold water and the extract collected in test tubes as were the extracts of the cell wall proper. Next the debris was washed with ethanol and ethyl ether. These extracts were discarded. Extraction in sequence was as follows: A. Twice for 10 minutes each with 3 ml hot water at the temperature of a boiling water bath; B. Three times with 3 ml hot 0.05 *N* HCl for 20 minutes each; C. Once with 3 ml 4 % NaOH or 5 % KOH at room temperature for 2 hours, followed by water rinse; D. Once with 2 ml 17.5 % NaOH or 24 % KOH overnight followed by water rinse, and E. Once with 3 ml hot acetolyzing reagent which dissolves cellulose (17) for 20 minutes followed by 1 ml of the same reagent. Extracts were made to volume and mixed; aliquots were plated on stainless steel planchets. This was done directly in the case of the cold water, hot water, and HCl extracts. Alkali extracts were neutralized either beforehand or directly on the planchets with HCl. The acetolyzed extract was heated 40 minutes in a boiling water bath with one-third volume added water. The sulfuric acid of the acetolyzing reagent was eliminated by adding one-half volume 2 *N* barium acetate. The supernatant plus washings of the $BaSO_4$ were mixed, made to volume, and aliquots of the partial hydrolysates were plated on the stainless steel planchets. Direct plating of the acetolyzed extracts was also tried; although this process yielded essentially similar results it was less useful for very low activity solutions due to the water absorbed during long counting periods. Carbohydrate components of the various fractions were determined by paper chromatography.

Both a thin end window Geiger-Müller tube and a gas flow counter fitted with a micromil window (Nuclear-Chicago) in an atmosphere of Q-gas were used. To compare the tables more easily however, all data were corrected to the gas flow counter by means of a common carbon 14 standard. All counts were corrected for background and self absorption (to infinite thinness). The results are expressed as total cpm per 100 sections. All treatments were carried out in triplicate; the average range was less than 15 per cent of the mean.

Total $C^{14}$ absorbed was obtained by adding all the fractions together with the cold water extract which contains the bulk of the cytoplasmic proteins and soluble components. Preliminary examination of these cold water extracts indicated the presence not only of radioactive glucose but also of radioactive protein, amino acids, and soluble polysaccharides. This total of course did not include $C^{14}O_2$ respired nor the discarded extracts. The latter amounts are small and can be neglected. The $C^{14}O_2$ respired is larger and conceivably might vary among the treatments although it probably varies less than does the uptake in the cold water extracts since respiration in Avena coleoptile sections is little affected by water stress (11).

Internal OP (corrected for section length) was determined by the plasmolytic method to the nearest 0.05 M for some of the treatments and was estimated for the rest. Estimates of OP for tissue in salt-free mannitol pretreatments are based on OP measurements of initial sections, on the negligible rise in OP due to mannitol (11), and on the section length in a given treatment. OP, DPD, and TP are expressed in terms of molarity of mannitol solutions. TP was determined by subtracting DPD from the measured or estimated OP.

Where growth potential was investigated experiments were carried out with 5 mg per liter of K-free indoleacetic acid (IAA).

## Results

Comparing high DPD non-salt treatment with high DPD salt treatment helps to clarify the relative roles of TP and OP in high DPD inhibition of growth and metabolism. If an increase in internal OP is primarily responsible for high DPD effects the salt treatment should enhance the high DPD induced effects due to absorption of ions. If on the other hand, TP is the determining factor, the salt treatment should decrease the high DPD induced effects due to an increase in TP.

In the earlier paper (10) treatment with 0.16 M KCl resulted in levels of $C^{14}$ incorporation which were markedly less than expected. Turgor effects on metabolism occurred despite the lower levels of incorporation. To check this point further, an experiment was carried out in which the following treatments were compared:

I. High DPD (equivalent to 0.35 M) treatment in which sections were pretreated 8 hours in 0.35 M mannitol and then transferred to 0.35 M mannitol with glucose-$C^{14}$. Since internal OP was found to be equivalent to 0.45 M mannitol, TP would be 0.10 M.

II. High DPD salt treatment in which sections were pretreated 8 hours in 0.175 M KCl (equivalent to 0.35 M mannitol) and then transferred to 0.35 M mannitol with glucose-$C^{14}$. Internal OP was measured and found to be equivalent to 0.60 M mannitol after 5 hours; therefore, TP would be in excess of 0.25 M after 8 hours. Since the work of Cooil and Bonner (5) indicates that previously absorbed Ca can be partially lost from coleoptile sections during subsequent treatment in water, an analysis of K before and after mannitol incubation was carried out for a salt treatment. It was found that no K was lost, and consequently it may be presumed that internal OP remained constant.

III. High DPD salt treatment similar to the one above, but in which the sections were transferred after pretreatment to 0.175 M KCl with glucose-$C^{14}$. This treatment was included to determine the effect of continuous presence of KCl at the same DPD as in the above treatments. The OP and TP here are probably slightly greater during the incubation with $C^{14}$.

These treatments (results summarized in table I) show that there is no marked difference in the total $C^{14}$ absorbed. Compared with non-salt treated sections, there are only small relative increases in incorporation into the total wall and into the hot acid extract of sections transferred from KCl to man-

TABLE I

EFFECT OF KCl AT CONSTANT DPD ON INCORPORATION OF $C^{14}$ FROM GLUCOSE-$C^{14}$ INTO CELL WALL FRACTIONS OF AVENA COLEOPTILE SECTIONS

| TREATMENT | CPM × $10^{-3}$ PER 100 SECTIONS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CELL WALL FRACTIONS | | | | | | | |
| | HOT $H_2O$ SOLUBLE | HOT HCl SOLUBLE | 4 % NaOH SOLUBLE | 17.5 % NaOH SOLUBLE | CELLULOSE | TOTAL | COLD $H_2O$ EXTRACT | TOTAL ABSORBED |
| High DPD, no salt* | 2.4 | 30.2 | 12.0 | 2.2 | 1.5 | 48.3 | 169 | 217 |
| High DPD, salt** | 2.8 | 33.4 | 24.5 | 4.7 | 4.5 | 69.9 | 161 | 231 |
| High DPD, salt*** | 1.6 | 23.9 | 18.3 | 3.6 | 2.0 | 49.4 | 171 | 220 |

8 hour pretreatment at 26° and 3 hour incubation with $C^{14}$ at 28.5° C. 1 microcurie glucose-$C^{14}$ per treatment.

* 0.35 M mannitol pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, estimated OP = 0.45 M, estimated TP = 0.10 M).

** 0.175 M KCl pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, estimated OP > 0.60 M, estimated TP > 0.25 M).

*** 0.175 M KCl pretreatment followed by 0.175 M KCl incubation with $C^{14}$. (DPD = 0.35 M, estimated OP > 0.60 M, estimated TP > 0.25 M).

nitol but there are two- to three-fold increases in alkali soluble fractions and the cellulose. The continued presence of high KCl concentrations during the $C^{14}$ incubation, however, interferes with cell wall metabolism in some as yet unclear fashion. This is particularly noticeable in the lower incorporation into the hot water and acid extracts.

Since there appears to be an inhibiting effect due to the high amounts of KCl present, another experiment was carried out using 0.025 M KCl plus 0.3 M mannitol in the salt pretreatment (instead of 0.175 M KCl). This was found to produce OP of 0.60 M and TP of 0.25 M. The high DPD, non-salt treatment was the same as above. Comparing high DPD salt treatment with intermediate DPD non-salt treatment of the same TP further clarifies the respective roles of TP and OP. Since TP would be similar in these two treatments, differences observed would be nil if TP effects are all-decisive. Any difference observed would have to be accounted for by OP differences in tissues of the two treatments and, possibly, by specific salt effects. Since unpublished data show that previous low turgor allows less subsequent growth at higher turgor than is found in tissue never exposed to low turgor, it was considered desirable to expose the sections of both these treatments to the same turgor history. Since the salt pretreated tissue appears to achieve a TP after 8 hours equal to one existing in a tissue whose DPD is equivalent to 0.15 M mannitol, as determined both by length of section and by estimate of internal OP, an intermediate DPD non-salt treatment was carried out in which tissue was placed in 0.35 M mannitol and the DPD was lowered to 0.15 M by adding known volumes of water during the 8 hour pretreatment. The internal OP being 0.4 M, the TP would be 0.25 M. After such pretreatment, sections were placed in 0.15 M mannitol with glucose-$C^{14}$.

The data obtained are presented in table II. Compared with the high DPD non-salt treatment, the intermediate DPD treatment shows about one-third more $C^{14}$ absorbed and two to three times as much $C^{14}$ in the non-cellulosic polysaccharide fractions and four times as much in the cellulose. The salt-treated sections show essentially as much total absorption of $C^{14}$ and incorporation into the non-cellulosic polysaccharide fractions as does the intermediate DPD-treated material, but relatively less incorporation into cellulose.

NaCl pretreatments, both at 0.175 M and at 0.025 M with 0.3 M mannitol, were also carried out in the same experiment. Internal OP of the lower concentration NaCl was found to be slightly less than for KCl giving rise to an apparently lower TP. Eight hour pretreatments were used; they were followed by incubation with glucose-$C^{14}$ and 0.35 M mannitol as above. As shown in the lower part of table II, salt treatment with NaCl also results in increased incorporation into all fractions except that the high NaCl pretreatment seems to have had relatively little effect on absorption of $C^{14}$ or on incorporation into the hot water soluble or alkali soluble fractions compared with the high DPD non-salt treatment. The lower NaCl with mannitol pretreatment, like the comparable KCl treatment, definitely enhances incorporation into all fractions. The difference in TP may be more apparent than real since the KCl tissue OP was actually calculated as 0.58 M while the NaCl tissue OP was calculated as 0.56 M. These differences are smaller than the experimental error.

To see if chloride ion was causing a specific effect on incorporation an experiment was carried out with another salt, namely $KNO_3$. The intermediate DPD non-salt treatment, however, remained at 0.15 M mannitol throughout the experiment. It can be seen

TABLE II

EFFECT OF KCl AND OF NaCl ON INCORPORATION OF $C^{14}$ FROM GLUCOSE-$C^{14}$ INTO WALL FRACTIONS OF AVENA COLEOPTILE SECTIONS

| TREATMENT | CPM × $10^{-3}$ PER 100 SECTIONS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CELL WALL FRACTIONS | | | | | | | |
| | Hot $H_2O$ soluble | Hot HCl soluble | 5 % KOH soluble | 24 % KOH soluble | Cellulose | Total | Cold $H_2O$ extract | Total absorbed |
| High DPD, no salt* | 2.5 | 16.2 | 10.3 | 7.2 | 2.1 | 38.2 | 154 | 192 |
| Intermediate DPD** | 3.0 | 40.5 | 24.3 | 10.9 | 7.9 | 86.6 | 221 | 308 |
| High DPD, salt*** | 4.8 | 42.3 | 23.9 | 9.9 | 5.2 | 86.1 | 213 | 299 |
| High DPD, salt† | 2.5 | 28.7 | 11.1 | 7.9 | 4.5 | 54.6 | 157 | 212 |
| High DPD, salt†† | 4.7 | 38.9 | 27.6 | 11.5 | 6.4 | 89.1 | 208 | 297 |

8 hour pretreatment at 26° C and 3 hour incubation with $C^{14}$ at 25° C. 1 microcurie glucose-$C^{14}$ per incubation treatment.

* 0.35 M mannitol pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, estimated OP = 0.45 M, estimated TP = 0.10 M).

** 0.35 M mannitol diluted with $H_2O$ after 2 hours to 0.3 M, after 4 hours to 0.2 M and after 6 hours to 0.15 M in pretreatment followed by 0.15 M mannitol incubation with $C^{14}$. (DPD = 0.15 M, estimated OP = 0.40 M, estimated TP = 0.25 M).

*** 0.025 M KCl + 0.3 M mannitol pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, OP = 0.60 M, TP = 0.25 M).

† 0.175 M NaCl pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, estimated OP = 0.60 M, estimated TP = 0.25 M).

†† 0.025 M NaCl + 0.3 M mannitol pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, OP = 0.55 M, TP = 0.20 M).

again that incorporation into the various fractions is increased by means of salt pretreatments (table III). Potassium nitrate appears to be less effective than KCl in restoring incorporation responses. This may possibly be due to slightly lower OP (found to be 0.55 M giving TP = 0.20) than for KCl treatment.

In order to see what sugars of the wall fractions are affected by the treatments, fractions were studied chromatographically. The hot water-soluble, dilute acid-soluble, and dilute alkali-soluble fractions of tissue (from an experiment similar to that described in table II) were hydrolyzed with 1 *N* $H_2SO_4$ for 5 to 6 hours. After removing excess $H_2SO_4$ with $BaCO_3$, the sugars were extracted with hot pyridine. The sugars were then chromatographed both in water saturated phenol and in butanol : acetic acid : water (4 : 1 : 5). The sugars were tentatively identified by position and aniline phthalate spray compared with sugar standards run at the same time. It was found that the hot water extract of polysaccharides appears to contain mainly glucose and glucose-like substances with small quantities of xylose, arabinose, and uronic acid; the dilute HCl extract polysaccharides contain mostly xylose and arabinose in about equal amounts with smaller quantities of glucose, galactose, and uronic acid. The dilute alkali extract polysaccharides consist of xylose, with smaller quantities of glucose, galactose, and arabinose. All these sugars were radioactive as shown by exposure to Ilford medical X-ray film. The completely hydrolyzed cellulose extract shows only glucose. Bishop et al (3), however, (by much more detailed chemical analysis) have shown minor amounts of other sugars to be present in the cellulose. Sugar spots were cut out of the chromatograms and counted under the micromil window. The semi-quantitative data obtained indicated that low turgor results in apparently less radioactivity in all sugars.

In order to examine the sugars of the concentrated alkali extracts a different method of hydrolysis was used. The alkali extracts (experiment described in table III) were treated with 83 % ethanol (final concentration) to precipitate polysaccharides. These precipitates were washed with ethanol and absolute ethanol. The dry precipitate was then hydrolyzed with 1 *N* HCl in sealed ampules for 8 hours (3). The dilute alkali extract yielded results as described above for the pyridine extractions. The concentrated alkali extract showed that mainly glucose polymers had been extracted with much smaller amounts of polymers of xylose and arabinose. All these sugars were also radioactive.

Since an increase in DPD causes a marked decline in IAA-induced elongation (11), a comparison was made between elongation and cell wall metabolism. A direct comparison of the above data with IAA-induced elongation is not possible since the DPD values involved appear to be above those where one can measure elongation after a short time period. The data of Ordin et al (11) indicated that one cannot detect elongation at a DPD equivalent to about 0.2 M mannitol when no absorbable solute is present. To make

TABLE III

EFFECT OF $KNO_3$ AND OF KCl ON INCORPORATION OF $C^{14}$ FROM GLUCOSE-$C^{14}$ INTO CELL WALL FRACTIONS OF AVENA COLEOPTILE SECTIONS

| TREATMENT | CPM × $10^{-3}$ PER 100 SECTIONS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CELL WALL FRACTIONS | | | | | | | |
| | HOT $H_2O$ SOLUBLE | HOT HCl SOLUBLE | 5 % KOH SOLUBLE | 24 % KOH SOLUBLE | CELLULOSE | TOTAL | COLD $H_2O$ EXTRACT | TOTAL ABSORBED |
| High DPD, no salt* | 1.7 | 13.7 | 6.3 | 5.6 | 1.2 | 28.5 | 107 | 135 |
| Intermediate DPD** | 2.7 | 38.2 | 20.0 | 7.8 | 6.6 | 75.3 | 204 | 279 |
| High DPD, $KNO_3$*** | 3.3 | 21.0 | 16.9 | 6.3 | 2.2 | 49.7 | 151 | 201 |
| High DPD, KCl† | 2.5 | 25.0 | 18.7 | 7.2 | 2.7 | 56.1 | 139 | 195 |

8 hour pretreatment and 3 hour $C^{14}$ incubation treatment at 27° C. 1 microcurie glucose-$C^{14}$ per incubation treatment.

* 0.35 M mannitol pretreatment followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, estimated OP = 0.45 M, estimated TP = 0.10 M).

** 0.15 M mannitol pretreatment followed by 0.15 M mannitol incubation with $C^{14}$. (DPD = 0.15 M, estimated OP = 0.40 M, estimated TP = 0.25 M).

*** 0.025 M $KNO_3$ + 0.3 M mannitol followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, OP = 0.55 M, TP = 0.20 M).

† 0.025 M KCl + 0.3 M mannitol followed by 0.35 M mannitol incubation with $C^{14}$. (DPD = 0.35 M, OP = 0.60 M, TP = 0.25 M).

the required comparison, therefore, it is necessary to carry out experiments in the range of DPD 0 to 0.2 M mannitol, i.e., where one can measure elongation.

For the $C^{14}$ experiments IAA was omitted since, as was shown earlier (12, 13), IAA causes small increases in incorporation of glucose-$C^{14}$, particularly into cellulose, only when elongation actually occurs. For the elongation experiments IAA was added and glucose-$C^{14}$ was omitted. Details of the treatments and results are given in table IV. Based on the same OP of 0.4 M, the tissue of the zero DPD treatment would have a TP equivalent to about 0.4 M and the

TABLE IV

EFFECT OF VARIOUS OSMOTIC TREATMENTS ON INCORPORATION OF $C^{14}$ FROM GLUCOSE-$C^{14}$ AND ON AUXIN-INDUCED ELONGATION

| TREATMENT | CPM × $10^{-3}$ PER 100 SECTIONS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CELL WALL FRACTIONS | | | | | | | | |
| | HOT $H_2O$ SOLUBLE | HOT HCl SOLUBLE | 5 % KOH SOLUBLE | 24 % KOH SOLUBLE | CELLULOSE | TOTAL | COLD $H_2O$ EXTRACT | TOTAL ABSORBED | ELONGATION* MM |
| Intermediate DPD** | 4.7 | 46.6 | 14.6 | 7.2 | 14.1 | 87 | 229 | 316 | 0.00 |
| Low DPD*** | 5.8 | 71.6 | 24.8 | 8.8 | 25.3 | 136 | 221 | 357 | 0.24 |
| Zero DPD† | 5.5 | 60.0 | 19.8 | 8.9 | 29.7 | 124 | 230 | 354 | 0.40 |
| Intermediate DPD, salt†† | 4.6 | 59.8 | 21.9 | 9.4 | 20.8 | 116 | 254 | 370 | 0.12 |

4 hour pretreatment at 25° C and 3 hour incubation at 26° C. 1 microcurie glucose-$C^{14}$ per incubation treatment without IAA or 5 mg per liter IAA per incubation without $C^{14}$.

* Elongation measured as net above minus IAA control.

** 0.2 M mannitol pretreatment followed by 0.2 M mannitol incubation with $C^{14}$. (DPD = 0.2 M, estimated OP = 0.40 M, estimated TP = 0.20 M).

*** 0.2 M mannitol diluted with water after 2 hours to 0.1 M and after 3 hours to 0.05 M in pretreatment followed by 0.05 M mannitol incubation with $C^{14}$. (DPD = 0.05 M, estimated OP = 0.40 M, estimated TP = 0.35 M).

† No solutes in either pretreatment or incubation except for $C^{14}$ in latter. (DPD = 0, OP = 0.4 M, TP = 0.4 M).

†† 0.025 M KCl + 0.15 M mannitol pretreatment followed by 0.2 M mannitol incubation with $C^{14}$. (DPD = 0.2 M, estimated OP = 0.5 M, estimated TP = 0.3 M).

tissue of the intermediate DPD treatment (equivalent to 0.2 M) would have a TP equivalent to 0.2 M. The OP of the tissue of the diluted mannitol treatment (low DPD = 0.05 M) would be about 0.4 M giving rise to a TP of about 0.35 M. After 4 hours the sections of both this treatment and of the intermediate DPD (equivalent to 0.2 M) salt treatment had the same length. This would indicate approximately equal TP. An estimate, however, of the OP of the tissue of the intermediate DPD salt treatment, which was not measured here, indicates that it possibly was about 0.5 M giving rise to a TP of about 0.3 M. A longer period of salt absorption with consequently higher TP would have been more desirable since it would have permitted a better evaluation of the effect of internal OP.

The salt-treated sections grew about half as much as the sections of the low DPD treatment, probably because of the lower TP of the former. The small increase in OP involved here would have no influence on elongation as indicated earlier (11). The sections of the low DPD treatment grew less than those of the zero DPD treatment. The intermediate DPD treatment depressed absorption and incorporation into the various cell wall fractions, excluding cellulose, 10 to 25 per cent (depending upon the fraction) compared with the zero DPD treatment. Incorporation into cellulose was depressed to about half. The low DPD treatment, compared with the zero DPD treatment, tended to stimulate incorporation into most of the fractions except for cellulose into which incorporation was less. Incorporation into the wall of the salt-treated sections showed little difference from the zero DPD-treated sections except that again incorporation into cellulose was less. Due to the possible TP difference between the salt and low DPD treatments one cannot judge directly if any part of the lesser cellulose incorporation in the salt treatment is due to a higher OP. In view of the lesser incorporation into the other fractions, however, it can be inferred that OP probably had little inhibiting influence in this range of DPD. Taking into account that a certain amount of incorporation into cellulose can occur when the potential for elongation is nil, the changes in cellulose appear to vary in the same fashion as rates of elongation. This is not the case for the other fractions.

## Discussion

That the incorporation studied here appears to be net synthesis rather than turnover is suggested by the earlier work of Ordin et al (13) where little loss of previously incorporated $C^{14}$ occurred from tissue during a long period of incubation in 0.09 M nonradioactive glucose.

The results presented earlier (10) indicate that in the Avena coleoptile, metabolism of the various cell wall fractions is greatly dependent upon the DPD of the tissue when little or no solute absorption can occur. A relatively small DPD (in the absence of salts) brings about a notable decrease in incorporation of $C^{14}$ from glucose into the hot acid soluble fraction and into the cellulose; the other non-cellulosic polysaccharides show a marked decline only after further increases in DPD which bring the tissue near the state of incipient plasmolysis.

The absorption of salts results in increased metabolism and elongation for a given DPD, and except when high salt concentrations are used, does not seem to have marked toxic effect. The salt absorption results in both increased OP and TP.

That an increase in internal OP might directly affect enzymes is indicated by Tyler (19), who showed that an increase in OP can decrease succinoxidase activity from animal tissue in a manner not explainable on the basis of affecting mitochondrial membrane structure. Thus dehydration may affect enzymes either directly or indirectly. The increase in internal OP is not a depressing factor in the metabolism of non-cellulosic polysaccharides since $C^{14}$ is incorporated into these substances as readily when the internal OP and TP are high, as when the OP is lower but TP is the same. The lower incorporation into cellulose in salt treated sections than in varying DPD treated sections of equal TP, in the 0.15 M to 0.35 M DPD range, may be due either to OP differences or possibly, to specific salt effects. Specific salt effects appear to be partly ruled out from the similarity of results for different salts.

This investigation indicates that turgor is the chief governing osmotic influence on both cell wall metabolism and elongation. In tissue in which TP has increased from a low value to a high one (0.35 M to 0.4 M TP) there is a tendency for the metabolism of the non-cellulosic polysaccharides to be accelerated compared with that in tissue maintained at the same high turgor.

The problem of interaction of elongation and synthesis of cell wall material is an intricate one. Total cell wall synthesis is not a primary factor of the auxin mechanism, but does appear to increase when elongation occurs at room temperature as shown by Bonner (2). According to the results presented here, both rate of elongation and a portion of the cellulose synthesis are affected by turgor in the same fashion. In view of this and the work (12) concerning the effect of galactose on both elongation and cellulose metabolism, it is tempting to suggest that the elongation response to water stress may be due, in part, to an effect on cellulose. As indicated earlier (13) cellulose is not directly affected by auxin. At full turgor, in the absence of auxin, there would be slightly less cellulose synthesis than with auxin; synthesis which did occur would be expected to thicken the cell wall. In the presence of mannitol there would be considerably less cellulose synthesis and one would expect much less cell wall thickening than at full turgor without auxin. Although Bayley and Setterfield (1) suggested, on the basis of microscopic evidence, that no thickening of cell walls occurs in non-elongating

Avena coleoptiles, in the presence of mannitol, the weight measurements of the cell walls indicate a trend for the more slowly elongating tissue to have more mass per unit length. Unfortunately, the microscopic evidence does not compare elongating with non-elongating tissue at either the same DPD or TP. Comparisons under the microscope of non-elongating tissue at different values of TP would also have been desirable.

Other processes which are inhibited by water stress are the non-auxin-induced methyl esterifications of the hot water and hot HCl soluble constituents of Avena coleoptiles (14). In part, these presumably accompany the inhibition of polysaccharide synthesis.

The overall effects on incorporation definitely indicate that a positive pressure of cytoplasm on the cell wall is required to maintain synthetic reactions, but it is difficult to visualize just why the degree of pressure should matter. A recent paper by Schaefer (16) indicates that in Elodea leaves and Lemna minor roots the adhesion between cytoplasm and cell wall begins to decrease in the hypotonic region. This may be the cause of the reduced incorporation as well as of reduced rate of elongation. Both the general and the differential effects on rates of incorporation into the various fractions found in this investigation may be due to effects on degree of contact and on strategic juxtaposition of the specific enzymes and substrates involved, some of the required substances being in the cytoplasm and others presumably in the wall itself.

The conclusions reached here cannot be applied to other plants (or even possibly to later stages in the same plant) without further investigation. In fact, in a recent paper Nezgovorova (9) found that $C^{14}O_2$ is incorporated into the cell wall material of corn and Avena plants more rapidly in dry soil than in wet soil. Unpublished data from this laboratory, however, indicate that greenhouse-grown sunflower plants show less incorporation into cell walls of the leaves if the plants are exposed to soil moisture stress at the time of the incubation with glucose-$C^{14}$. Long-term experiments may give other results due to the effect of previous history on the responses examined here and on other factors which are of importance only in the long run.

Direct comparison of tissue exposed to stress due to soil moisture tension and that exposed to equal stress due to mannitol solutions is not possible, since soil DPD bears a more indirect relation to the plant DPD than does mannitol solution DPD. Gingrich and Russell (7) showed that tension in the soil has greater depressing effect than corresponding OP of solution culture on corn seedling root growth and dry matter production. This is due presumably to the movement of $H_2O$ in unsaturated media being slower than in aqueous solutions. Thus the actual stress at root surfaces could be much greater than that measured in the soil mass.

## Summary

A period of salt absorption by Avena coleoptile sections in solutions with high diffusion pressure deficits causes an increase in turgor and an increase in incorporation of $C^{14}$ from glucose-$C^{14}$ into all fractions of the cell wall in experiments of short duration. The increases in incorporation are similar to those obtained when turgor is increased by reducing diffusion pressure deficit.

Very high salt concentration pretreatments restore incorporation rates less than do lower salt pretreatments.

All the sugar components of the cell wall fractions become radioactive and appear to be affected by treatments in the same general way as the total activity of the fractions concerned.

In sections in which turgor pressure has increased from a low value to one approaching full turgor (0.4 M) there is a tendency for the metabolism of the acid and dilute alkali soluble wall constituents to be stimulated compared with that in sections maintained at constant full turgor.

The rate of elongation and amount of incorporation of $C^{14}$ into cellulose show rather similar patterns of behavior with respect to effect of osmotic factors.

It is suggested that turgor is the chief component of diffusion pressure deficit affecting elongation and incorporation into the cell wall and that some aspect of cellulose synthesis may be involved in the elongation response to turgor in Avena coleoptiles.

## Literature Cited

1. Bayley, S. T. and G. Setterfield. 1957. The influence of mannitol and auxin on growth of cell walls in Avena coleoptiles. Ann. Bot. 21: 633–641.
2. Bonner, J. 1934. Studies on the growth hormones of plants. V. The relation of cell elongation to cell wall formation. Proc. Nat. Acad. Sci., U. S. 20: 393–397.
3. Bishop, C. T., S. T. Bayley and G. Setterfield. 1958. Chemical constitution of the primary cell walls of Avena coleoptiles. Plant Physiol. 33: 283–289.
4. Cleland, R. and J. Bonner. 1956. The residual effect of auxin on the cell wall. Plant Physiol. 31: 350–354.
5. Cooil, B. J., and J. Bonner. 1957. The nature of growth inhibition by calcium in the Avena coleoptile. Planta 48: 696–723.
6. Crafts, A. S., H. B. Currier, and C. R. Stocking. 1949. Water in the Physiology of Plants. Chronica Botanica, Waltham.
7. Gingrich, J. R. and M. B. Russell. 1957. A comparison of effects of soil moisture tension and osmotic stress on root growth. Soil Sci. 84: 185–194.
8. Kertesz, Z. I. 1951. The Pectic Substances. Interscience Publishers, New York.
9. Nezgovorova, L. A. 1957. Effect of water relations in plants on the uptake and distribution of carbon during photosynthesis. Fiziologiia Rastenii 4: 440–448.

10. Ordin, L. 1958. The effect of water stress on the cell wall metabolism of plant tissue. In: Radioisotopes in Scientific Research. Vol. IV. Pp. 553–564. Pergamon Press, London.
11. Ordin, L., T. H. Applewhite, and J. Bonner. 1956. Auxin-induced water uptake by Avena coleoptile sections. Plant Physiol. 31: 44–53.
12. Ordin, L. and J. Bonner. 1957. Effect of galactose on growth and metabolism of Avena coleoptile sections. Plant Physiol. 32: 212–215.
13. Ordin, L., R. Cleland, and J. Bonner. 1955. Influence of auxin on cell wall metabolism. Proc. Nat. Acad. Sci., U.S. 41: 1023–1029.
14. Ordin, L., R. Cleland, and J. Bonner. 1957. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32: 216–220.
15. Richards, L. A. and C. H. Wadleigh. 1952. Soil water and plant growth. In: Soil Physical Conditions and Plant Growth, B. T. Shaw ed. Pp. 73–251. Academic Press, New York.
16. Schaefer, G. 1958. Über Veränderungen des mechanischen und biologischen Kontaktes von Plasma und Zellwand in subplasmolytischen Konzentrationen von Anelektrolyten. Planta 51: 414–439.
17. Schramm, M. and S. Hestrin. 1954. Synthesis of cellulose by *Acetobacter xylinum*. I. Micromethod for the determination of celluloses. Biochem. Jour. 56: 163–166.
18. Slatyer, R. O. 1957. The significance of the permanent wilting percentage in studies of plant and soil water relations. Bot. Rev. 23: 585–636.
19. Tyler, D. B. 1954. The effect of osmotic pressure on succin-oxidase activity. Jour. Biol. Chem. 209: 893–900.
20. Wadleigh, C. H. and H. G. Gauch. 1948. Rate of leaf elongation as affected by the intensity of the total soil moisture stress. Plant Physiol. 23: 485–495.

# AN ARTIFACT IN CHROMATOGRAPHY OF INDOLIC AUXINS[1, 2, 3]

COLETTE NITSCH[4] AND J. P. NITSCH[4]

Department of Floriculture and Ornamental Horticulture, Cornell University, Ithaca, N. Y.

During investigations on the chromatographic separation of natural auxins an artifact was discovered which could be a cause of many errors. In extracts of grapes, peaks of biological activity appeared on paper chromatograms at the position of 3-indolylacetonitrile (IAN) and the ethylester of 3-indoleacetic acid (IAE) (*2, 3*). In new experiments with Concord grapes (*5*) peaks of biological activity were found again at the same Rf's as IAN and IAE. These regions were cut out, eluted from the paper, and the eluates re-chromatographed separately. Each of the two new chromatograms always showed at least two peaks of activity located at the IAN and IAE positions, which suggested an interconversion between the two active auxins. A similar apparent interconversion was reported for the water-soluble growth substances extracted from tomato roots (*1*). The apparent interconversion occurring between the auxins of the grape extracts, however, existed only when a control paper strip, spotted with synthetic IAN and IAE, was present together with the strip spotted with the extract in the same chromatographic tube. A thorough investigation of this situation was undertaken.

## Methods

Ascending chromatography in the dark was used throughout. Two-centimeter wide strips of Whatman No. 3 MM paper were hung in large tubes according to a technique described previously (*2, 3*). One hundred ml of solvent was poured into each 65 × 500 mm chromatographic tube. It has been shown (*2, 3*) that hexane and other water-insoluble hydrocarbons can separate neutral auxins such as IAN and IAE, provided enough water impregnates the fibers of the paper. To increase the amount of water present in the solvent, a mixture of petroleum ether (bp 35–70° C), chloroform and water was used. It gave reproducible Rf's which could be modified at will by changing the ratio of petroleum ether to chloroform, the water added being always 10% (v:v). Thus, when this ratio was 85:5, the Rf's of IAN and IAE were 0.15 and 0.55, respectively, as compared with 0.30 and 0.70 when the ratio was 80:10, and 0.45 and 0.85 when it was 75:15. Unless indicated otherwise all the paper strips were equilibrated overnight (about 15 hrs) over the solvent (at 25° C) before actually running the chromatograms.

[1] Received for publication September 28, 1959.
[2] Work supported by grant RG-4840 from the National Institutes of Health, Bethesda, Md., and by grant G-4046 from the National Science Foundation, Washington, D. C.
[3] Research reported at the Annual Meeting of the Northeastern Section of the American Society of Plant Physiologists held at Brookhaven National Laboratory in May 1957.
[4] Present address: Laboratoire du Phytotron, Gif-sur-Yvette (S.etO.), France.

In order to determine the location of the auxins on the paper, the oat first-internode test was used (4). The paper strips were cut into 20 equal segments each of which was then incubated with 10 first-internode sections of Brighton oats in test tubes containing 1 ml of pH 5.0 citrate-phosphate buffer (4). The tubes were rotated at 1 rpm at 25° C in the dark for 20 to 24 hours. At the end of this period, the first-internode sections were measured to the nearest 0.1 mm. The average elongation over the initial 4 mm length was plotted against Rf to give the histograms of figures 1 to 6. In these histograms any deviation from the controls (dotted lines) which is equal to or larger than 0.2 mm is statistically significant.

## Results

I. Effect of Control Strip in Chromatographic Tubes: Two blank paper strips were hung in clean tubes over fresh solvent. One tube contained the blank strip alone, the other contained, in addition, a control strip which had been spotted with a mixture of 10 μg each of 3-indolylacetic acid (IAA), 3-indolylbutyric acid (IBA), IAN, and IAE. After equilibration and chromatography in the petroleum ether solvent, the blank paper strips were dried in an air current in the hood and cut into 20 1-cm pieces for bioassay. The bioassay showed at least three auxin peaks on the blank paper which had been chromatographed with the control strip. These peaks were located at the Rf's where the synthetic auxins had moved on the control paper (fig 1, top). The highest auxin activity occurred at the IAN position. The blank paper which had been chromatographed without a control strip showed no biological activity (fig 1, bottom). These results were obtained over and over again. With the petroleum-ether solvent or a hexane : water (90 : 10 v/v) solvent, peaks of biological activity always appeared at the IAN and IAE positions when a control strip spotted with synthetic IAA, IAN, and IAE was chromatographed together with another strip. At the IAA position, however, biological activity was detected only rarely and remained very low.

The transfer of biological activity from one paper to another occurred also with solvents such as isopropanol : ammonia : water (80 : 10 : 10 v/v), but to a smaller degree. Thus, biological activity appeared on blank paper strips when they were chromatographed in the isopropanol solvent together with control strips spotted with a mixture of IAA (2 μg), IBA (1 μg), IAN (10 μg), and IAE (5 μg). The biological activity appeared at the IAN and IAE position. No activity could be detected on blank strips when controls were absent (fig 2). The *n*-butanol : ammonia : water solvent used by Britton et al (1) in their studies of the interconversion of auxins X, Y, and Z gave also indications of a movement of biological activity from one paper strip to another, but the amounts of auxins found on blank papers chromatographed with control strips were very low.

The experiments clearly indicate that each time a control strip spotted with synthetic indolic auxins was added to a tube containing a blank paper, some biological activity could be found on the blank chromatogram. This effect was generally most pronounced with IAN and with solvents containing hexane or petroleum ether.

II. Effect of Solvent During Equilibration: A possibility that suggested itself was that some of the auxins spotted on the control strips were in fact distilling over to the solvent in the tube, and that this solvent was then carrying the auxins to the blank strip. In order to check this possibility, a blank strip (A) (fig 3, top left) was first equilibrated over a petroleum ether : chloroform : water (80 : 10 : 10) solvent together with a control strip ($C_1$) spotted with IAA (2 μg) + IBA (1 μg) + IAN (8 μg) + IAE (4 μg). After about 15 hours the blank paper (which never touched the solvent) was taken out and rapidly transferred to another tube without a control strip. After 4 hours of re-equilibration in the second tube, the blank strip A was lowered into the solvent and chromatographed. Biological activity showed up on blank strip A, indicating that a transfer of auxins had taken place from the control strip $C_1$ to A during the first equilibration period and without the intermediary of the liquid phase of the solvent contained in the first tube.

To investigate this paper-to-paper transfer further, another experiment was set up in which a blank strip B was equilibrated with a control $C_2$ similar to $C_1$ in a tube without any solvent. After about 15 hours, strip B was transferred to a new tube containing the petroleum ether solvent, re-equilibrated for

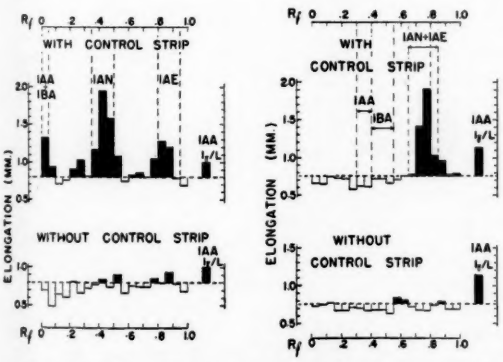



Fig. 1 (*left*). Histograms of two blank paper strips chromatographed in petroleum ether : chloroform : water (75 : 15 : 10) in the presence of a control strip spotted with synthetic auxins (*top*), or with no control strip (*bottom*).

Fig. 2 (*right*). Histograms of two blank paper strips chromatographed in isopropanol : ammonia : water (80 : 10 : 10). (*Top*) : control strip with synthetic auxins present in the same tube. (*Bottom*) : no control strip.

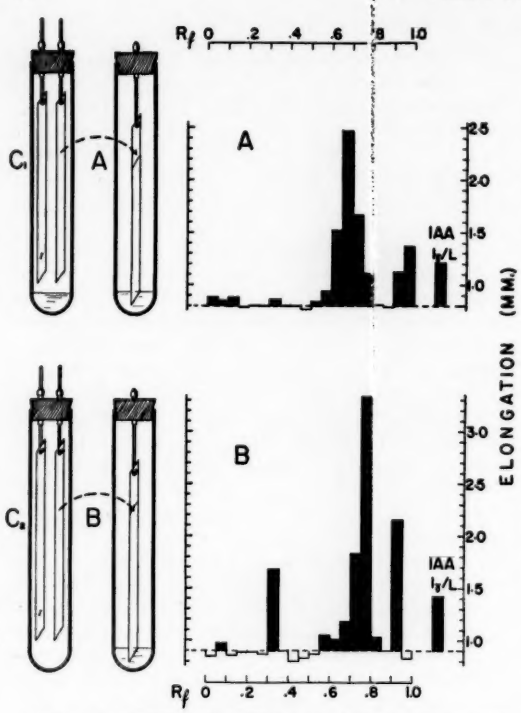



FIG. 3. Transfer of synthetic auxins from one paper to another during equilibration with (*top*) and without (*bottom*) a solvent in the equilibrating tube. $C_1$ and $C_2$: control papers spotted with synthetic IAA, IBA, IAN, and IAE. A and B: blank papers first equilibrated with the controls for 15 hours, then moved to a different tube for chromatography in petroleum ether: chloroform: water (80: 10: 10). The histograms show the biological activity found on the blank papers after chromatography.

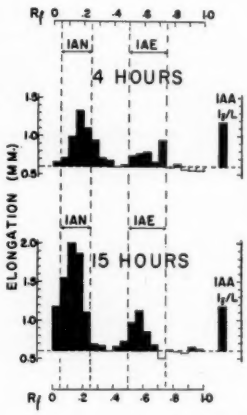
4 hours, and chromatographed. This blank strip also was found to have clear-cut biological activity (fig 3, bottom).

From these two experiments one can conclude that transfer of biological activity from a control paper strip spotted with indolic auxins to a blank paper occurs through the air, without an intermediary solvent. It seems to be a sort of distillation or sublimation from the spot of the control strip to the blank strip.

A point which remained a puzzle for a long time was the location of the biological activity on the blank strips: it occurred generally at the same Rf's as on spotted chromatograms. This effect was found to result from our habit of hanging the two strips parallel to each other. Thus the indolic compounds could move horizontally across the 3 to 4 cm-wide vapor phase during the equilibration period and impregnate the blank paper at the same position as that of the spot on the spotted strip. An experiment in which the blank strip was equilibrated with its lower end lower than that of the spotted strip resulted in a correspondingly higher Rf of the biological activity on this blank strip.

III. EFFECT OF SHIELDING: If there is a horizontal transfer of auxin from one paper strip to another, then placing a shield between the two strips should prevent it. This possibility[5] was tested in the following way: A 2-cm wide paper strip was spotted with 8 μg of IAN and hung in a chromatographic tube over hexane: chloroform: $H_2O$ (75: 15: 10) parallel with a blank strip. Between the two, a 4-cm wide paper strip (Whatman 3 No. MM) was interposed. After equilibration for about 15 hours at room temperature, the blank strip and the shield were bioassayed. The shield showed a definite growth-promoting area corresponding to the IAN which had moved horizontally from the spotted chromatogram. On the contrary, the blank strip behind the screen, had not picked up any biological activity. These results confirm the existence of a horizontal transf of substance through a vapor phase. The movin auxin is picked up by the first surface it hits. Protection from contamination by this process can be achieved by the use of a paper shield.

IV. EFFECT OF LENGTH OF EQUILIBRA PERIOD: Since the transfer of auxins from on



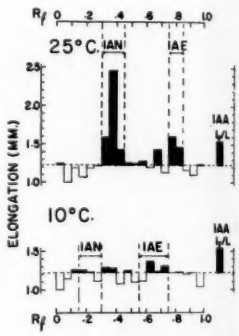




FIG. 4 (*left*). Effect of the duration of the equilibration period. Histograms of two blank paper strips chromatographed in petroleum ether: chloroform: water (85: 5: 10) together with a control strip spotted with IAA, IBA, IAN, and IAE. Equilibration times over the solvent: 4 hours (*top*) and 15 hours (*bottom*).

FIG. 5 (*right*). Effect of temperature. Histograms of two blank paper strips chromatographed in the presence of control strips spotted with IAA, IBA, IAN, and IAE in petroleum ether: chloroform: water (75: 15: 10) at 25° C (*top*) and 10° C (*bottom*).

[5] Suggested by Dr. E. M. Shantz.

ier to another occurs mainly during the equilibration period, an experiment was performed to find if the duration of the equilibration period had any effect upon the amount of auxins transferred. Two blank paper strips were equilibrated over petroleum : ether : chloroform : water (85 : 5 : 10) in two different tubes, each together with a control strip spotted with IAA (2 μg) + IBA (1 μg) + IAN (10 μg) + IAE (5 μg). The first strip was equilibrated for 4 hours, the second for 15 hours before chromatography. The results (fig 4) showed that transfer of IAN and IAE had occurred already after 4 hours of equilibration; the amounts of auxins transferred increased with time. A quantitative estimation indicated that there was ten times more IAN and IAE transferred after 15 hours than after 4 hours.

V. Effect of Temperature: If the process by which indolic auxins are transferred from one paper strip to another resembles a distillation, it must be affected by temperature. Two blank paper strips were chromatographed, each in a different tube and each with a control strip spotted with 10 μg each of IAA, IBA, IAN, and IAE. The solvent was petroleum ether + chloroform + $H_2O$ (75 : 15 : 10). The equilibration time was approximately 15 hours in both cases, but one tube was maintained at 25° C and the other at 10° C. The results are shown in figure 5. At 25° C, there was a clear-cut transfer of IAN and IAE, but not at 10° C. Similar results were obtained with isopropanol : ammonia : water (80 : 10 : 10). A fortiori, no transfer of IAN or IAE occurred when equilibration and chromatography were performed at the even lower temperature of 1° C.

VI. Effect of Used Solvent: Although it has been shown that most of the transfer of indolic auxins from one paper to another could occur in the absence of any solvent, one expects that if indole compounds can move from one paper to another, they can also distill from the paper to the solvent. To check this point, a blank strip was chromatographed in a petroleum ether-chloroform-water (80 : 10 : 10) mixture which had been used once previously to chromatograph three strips, each spotted with IAA (2 μg) + IBA (1 μg) + IAN (8 μg) + IAE (4 μg). There was no control strip in the tube during the chromatography of the blank strip. Biological activity was present on the blank strip (fig 6), whereas no such activity could be detected on similar blank chromatograms run in fresh solvent.

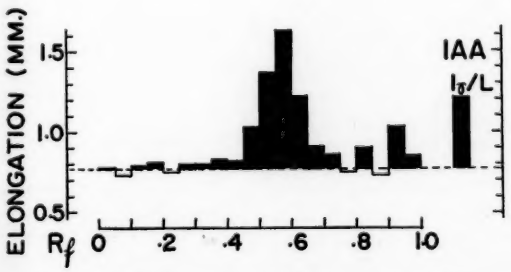



Fig. 6. Histograms of a blank paper strip chromatographed in petroleum ether : chloroform : water (80 : 10 : 10) without a control but in a solvent which had been used once before to run three chromatograms spotted with IAA, IBA, IAN, and IAE.

## Conclusions

These experiments show that misleading results may be obtained by running control strips spotted with synthetic indolic auxins together with other chromatograms (the control strips may lose some of the auxins to other chromatograms in the same tube). Among the auxins tested, IAN seemed to transfer with the greatest ease; IAE was second easiest, and IAA generally did not appear to be transferred. It is expected that other indolic compounds such as indole itself, could be transferred with even greater ease. Re-using solvents in which chromatograms have been run before (which is commonly done in many laboratories) is also a source of these artifacts.

In view of these results, we have re-examined some of our previously published data. The results of Nitsch and Nitsch (3) and Nitsch (2) in which plant extracts have been chromatographed in the presence of control strips spotted with IAA, IAN, and IAE have to be interpreted in the light of the present findings. Thus it is possible that the IAN and IAE claimed to have been found in Tokay grapes may actually be artifacts since these auxins were not detected in Concord grapes (5) when no control strips were present. The IAN and IAE peaks found in chromatograms of tissue cultures of *Parthenocissus tricuspidata* (2, fig 22) are most likely to be artifacts also. The consideration of these peaks as artifacts would actually make our previous results more logical, in that the difference between normal, habituated, and crown-gall tissues would lie in differences in the amounts of auxins located at Rf's 0.3 and 0.5 which are not artifacts.

To be certain to obtain chromatograms free of artifacts of the kind reported here, one should: A. Use one chromatogram per tube with the control strip in a separate tube, and B. Use fresh solvent in a clean tube each time. However, low temperatures (1 to 10° C), at which little transfer occurs, provide a means of running several chromatographic strips in the same tube, including a control with synthetic IAN and IAE, without the apparent transfer of compounds.

## Summary

Indolic auxins, especially 3-indolylacetonitrile and the ethyl ester of 3-indolylacetic acid, have been shown to move from one chromatographic paper strip to another. This transfer of active substances occurs during equilibration and is not dependent upon the presence of a solvent.

The transfer phenomenon is dependent upon temperature and the length of the equilibration period; it is particularly marked with solvents containing hexane or petroleum ether. Use of the same solvent repeatedly for several chromatographic runs also produces artifacts and should be avoided. Methods of circumventing these difficulties are described.

## Literature Cited


1. Britton, G., S. Housley, and J. A. Bentley. 1956. Studies in plant growth hormones. V. Chromatography of hormones in excised and intact roots of tomato seedlings. Jour. Exp. Bot. 7: 239–251.
2. Nitsch, J. P. 1955. Methods for the investigation of natural auxins and inhibitors. In: The Chemistry and Mode of Action of Plant Growth Substances, R. L. Wain, and F. Wightman, eds. Butterworths, London, 3–31.
3. Nitsch, J. P., and C. Nitsch. 1955. The separation of natural plant growth substances by paper chromatography. Beitr. Biol. Pflanz. 31: 387–408.
4. Nitsch, J. P. and C. Nitsch. 1956. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test for auxins. Plant Physiol. 31: 94–111.
5. Nitsch, J. P., C. Pratt, C. Nitsch, and N. J. Shaulis. 1960. Natural growth substances in Concord and Concord Seedless grapes in relation to berry development. Amer. Jour. Bot. 47: (in press).


# PHYSIOLOGICAL STUDIES ON NODULE-NITRATE REDUCTASE[1, 2]


GEORGE CHENIAE AND HAROLD J. EVANS[3]

Department of Botany and Bacteriology, North Carolina Agricultural Experiment Station, Raleigh, N. C.


A previous publication (9) reported the occurrence and some of the properties of a reduced diphosphopyridine nucleotide (DPNH) nitrate reductase associated with Rhizobium cells from nodules of several different legumes. Further studies on properties of the nitrate reductase system from soybean nodules (7) have shown that succinate, leuco-methylene blue, and reduced benzylviologen also will serve as electron donors. In addition, evidence has been obtained indicating that either menadione (2-methyl-1, 4-naphthoquionone), or a cofactor from Rhizobium cells, is necessary in this system for the transfer of electrons from DPNH to nitrate (5, 6, 7). When succinate is used as the electron donor, the cofactor is not required.

Physiological experiments (5) have indicated that the nitrate reductase activities of Rhizobium cells from nodules of soybean plants inoculated with different strains of *Rhizobium japonicum* are correlated positively with nitrogen-fixing efficiencies of the various strains. When soybean plants were inoculated with pure cultures of Rhizobia and grown aseptically in the absence of a source of combined nitrogen, they produced nodules with high nitrate reductase activity. As yet, the possible relationship, if any, of nodule-nitrate reductase to the process of nitrogen-fixation remains obscure. The possibility has been considered (5, 7) that the nodule-nitrate reductase is non-specific for oxidant, and that nitrate is not the natural substrate for the enzyme.

This manuscript describes some results of additional physiological experiments in which the effects of various factors on the specific activity of the succinate-nitrate reductase in nodules have been studied. In general, experiments have been designed to determine whether those factors reported to influence the fixation of nitrogen also influence the activity of the nodule enzyme.

## Materials and Methods

Culture Methods: Sand-culture was used in growing the leguminous plants for the experiments reported in tables I, II, III, and IV, and water culture for experiments 5 and 6 where deficiencies of soybean plants were investigated. Soybean seeds (*Glycine max* (L.) Merr. var. Lee) or seeds of other legumes indicated in table I were inoculated with commercial nitrogen-fixing inoculum and sown in flats of sand for producing seedlings for experiments 1, 2, 3, 4, and 5. Flats were supplied daily with half strength nitrogen-free nutrient solution (14). After 5 to 8 days, the seedlings were transferred to the culture vessels used in the various experiments. Soybean seedlings used for Mo deficiency experiments


[1] Received October 20, 1959.

[2] Published with the approval of the Director of the North Carolina Agricultural Experiment Station as Paper No. 1083.

[3] This investigation was supported in part by a Grant (No. G 2653) to Harold J. Evans from the National Science Foundation.

TABLE I

NITRATE REDUCTASE ACTIVITY OF BACTERIAL CELLS FROM NODULES OF LEGUMINOUS PLANTS

| LEGUME | ENZYME ACTIVITY* | |
|---|---|---|
| | WITHOUT SUCCINATE | WITH SUCCINATE |
| Peanut (*Arachis hypogaea*) | 3 ± 1 | 17 ± 2 |
| Cowpea (*Vigna sinensis*) | 76 ± 6 | 130 ± 7 |
| Velvetbean (*Stizolobium deeringianum*) | 26 ± 3 | 82 ± 6 |
| Crotolaria (*Crotolaria mucronata*) | 9 ± 5 | 21 ± 4 |
| Lespedeza (*Lespedeza stipulacea*) | 14 ± 6 | 60 ± 5 |
| Soybean (*Glycine max*) | 36 ± 4 | 242 ± 20 |

* Activity is reported as millimicromoles $NO_2^-$ formed in 10 minutes per mg protein. The values are means of three replications with standard deviations. For other experimental details see Materials and Methods.

were grown in sand which had been washed with hydrochloric and oxalic acids and then with sufficient demineralized water to remove the acid. Seedlings for this experiment were supplied with a half strength solution lacking molybdenum and nitrogen.

EXPERIMENT 1 (table I): Four to six plants of each species were grown in 3 gallon clay pots filled with quartz sand which previously had been washed with water. The nutrient solution was identical in composition with that used by Anderson and Evans (1) except that $CaCl_2$ was substituted for $Ca(NO_3)_2$ at an equivalent concentration. Throughout the growth period, plants in each culture vessel were supplied with 1 liter of nutrient solution for each of 2 successive days and then with 1 liter of water every 3rd day. Excess water or nutrient solution escaped through a drain.

The experiment was designed as a randomized block with three replications and was carried out in the greenhouse during the summer. The plants were harvested 8 to 10 weeks after planting.

EXPERIMENT 2 (table II): Soybean plants were grown in 3 gallon clay or polyethylene pots which were filled with a mixture of two parts of water-washed sand and one part crushed oyster shells (particles 5-10 mm diameter). Nodules were produced more abundantly in this medium than in sand alone. The nutrient solution was identical with nitrogen-free solution of Hoagland and Arnon (14) with the exception that iron was supplied in the form of hydrogen ferric ethylenediamine di(*o*-hydroxyphenyl acetate) (Geigy Chemical Co.). Nutrient solution was supplied as indicated in experiment 1.

Experiment 2 was arranged in a randomized block in the greenhouse. Four plants were grown in each culture vessel and each treatment was replicated three times. Plants were grown during the summer months and harvested after the periods indicated (table II).

EXPERIMENT 3 (table III): The culture technique was essentially identical with that of experiment 2. Velvet bean plants (*Stizolobium deeringianum*) were grown in the greenhouse during November, December, January, and February. Each treatment was replicated three times, and plants were harvested after the growth periods indicated in table III. To prevent premature flowering, plants were exposed daily to three 100-watt fluorescent lights between 5 and 7 AM and 5 and 11 PM.

EXPERIMENT 4 (table IV): In this experiment cultures without combined nitrogen were grown by the same general technique of experiment 2 except that five instead of four plants were grown in each pot. Certain culture treatments, indicated in table IV, included (in addition to the nitrogen-free solution) either $Ca(NO_3)_2$ or $(NH_4)_2SO_4$ at concentrations sufficient to supply 47 or 104 ppm N. The

TABLE II

EFFECT OF AGE OF SOYBEAN PLANTS ON NITRATE REDUCTASE ACTIVITY AND HEMOGLOBIN CONTENT OF NODULES AND DRY WEIGHT AND TOTAL N CONTENT OF TOPS*

| AGE OF PLANTS (WKS) | NITRATE REDUCTASE ACTIVITY (mμMOLES $NO_2$ /10 MIN/MG PROTEIN)** | HEMOGLOBIN CONC (μG/G FRESH NODULE) | DRY WT OF TOPS (G) | TOTAL N OF TOPS (%) |
|---|---|---|---|---|
| 3 | 52 ± 4 | 38 ± 14 | 0.8 ± 0.1 | 3.30 ± 0.14 |
| 6 | 86 ± 32 | 146 ± 5 | 5.0 ± 0.3 | 3.45 ± 0.14 |
| 9 | 242 ± 20 | 163 ± 3 | 17.9 ± 3.2 | 3.95 ± 0.07 |
| 11 | 129 ± 12 | 124 ± 18 | 35.0 ± 3.3 | 3.66 ± 0.06 |
| 12 | 136 ± 8 | 92 ± 8 | 36.2 ± 0.1 | 3.60 ± 0.14 |

* Plants were harvested after the growth periods indicated. All values reported are means with standard deviations of three replications.

** Nitrate reductase activities were determined during a 30-minute incubation period and calculated on a 10-minute basis. For other details of the experiment see Materials and Methods.

TABLE III

EFFECT OF AGE OF VELVET BEAN PLANTS ON NITRATE REDUCTASE ACTIVITY AND HEMOGLOBIN CONTENT OF NODULES AND DRY WEIGHT OF TOPS*

| AGE OF PLANTS (DAYS) | DRY WT (G) | HEMOGLOBIN CONTENT (μG/G FRESH NODULE) | NITRATE REDUCTASE ACTIVITY (MμMOLES NITRITE FORMED/10 MIN/MG PROTEIN) |
|---|---|---|---|
| 23 | 2.7 ± 0.4 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 36 | 4.8 ± 1.2 | 109 ± 34 | 171 ± 22 |
| 43 | 6.3 ± 1.0 | 101 ± 27 | 171 ± 6 |
| 64 | 31.2 ± 1.8 | 130 ± 13 | 138 ± 39 |
| 107 | 73.4 ± 1.1 | 0.0 ± 0.0 | 7 ± 5 |

* Values reported are means with standard deviations of three replications. For other details of the experiment see Materials and Methods.

experimental treatments (applied to four replicate cultures) were initiated 2 weeks after the seeds were planted. Plants were harvested after the growth periods indicated in table IV. The experiment was conducted during June, July, and August.

EXPERIMENTS 5A AND 5B (table V): In these experiments the influence of iron level on the growth and content of certain constituents in soybean plants was investigated. Five inoculated soybean seedlings (with cotyledons removed) were placed in 3 gallon polyethylene containers filled with the nitrogen-free nutrient solution used in experiment 2. The iron level, in the form of hydrogen ferric ethylenediamine di(*o*-hydroxyphenyl acetate), was varied as indicated in table V. Solutions were aerated and changed at intervals of approximately ten days. Experiments were arranged in randomized block designs, and each treatment in each experiment was applied to four replicate cultures. Plants from experiments 5A and 5B were harvested 41 days and 33 days, respectively, after seeds were planted. These experiments were conducted in the greenhouse during the summer.

EXPERIMENT 6 (table VI): In the experiment in which molybdenum deficient treatments were included, specially cleaned culture equipment and purified nutrient salts were utilized. Three-gallon polyethylene

TABLE IV

EFFECT OF ADDING COMBINED NITROGEN TO INOCULATED SOYBEAN PLANTS ON NITRATE REDUCTASE ACTIVITY AND HEMOGLOBIN CONTENT OF NODULES AND DRY WEIGHT OF TOPS*

| NITROGEN ADDED | | GROWTH PERIOD (DAYS) | NITRATE REDUCTASE ACTIVITY | HEMOGLOBIN | DRY WT |
|---|---|---|---|---|---|
| CONC (PPM) | COMPOUND | | (MμMOLES $NO_2^-$/10 MIN/MG PROTEIN) | (μG/G FRESH NODULE) | (G/CULTURE) |
| 0 | | 46 | 179 | 222 | 9.8 |
| 47 | $Ca(NO_3)_2$ | 46 | 116 | 184 | 11.3 |
| 104 | $Ca(NO_3)_2$ | 46 | 139 | 141 | 14.1 |
| 47 | $(NH_4)_2SO_4$ | 46 | 99 | 163 | 12.1 |
| 104 | $(NH_4)_2SO_4$ | 46 | 117 | 182 | 15.0 |
| 0 | | 79 | 135 | 142 | 32.2 |
| 47 | $Ca(NO_3)_2$ | 79 | 107 | 132 | 43.9 |
| 104 | $Ca(NO_3)_2$ | 79 | 61 | 134 | 52.8 |
| 47 | $(NH_4)_2SO_4$ | 79 | 119 | 116 | 46.4 |
| 104 | $(NH_4)_2SO_4$ | 79 | 107 | 129 | 55.8 |

* Values reported are means of determinations from four replicate cultures of each treatment. A statistical treatment of the data for nitrate reductase activities and hemoglobin contents revealed the following probabilities of significance for the comparisons indicated: (a) 0.0 N vs. all $NO_3^-$ levels at 46 days; $NO_3^-$ reductase 0.05, hemoglobin 0.05 (b) 0.0 N vs. all $NO_3^-$ treatments at 79 days; $NO_3^-$ reductase 0.01, hemoglobin N.S. (c) 0.0 N vs. all $NH_4^+$ treatments at 46 days; nitrate reductase 0.01, hemoglobin N.S. (d) 0.0 N vs. all $NH_4^+$ treatments at 79 days, $NO_3^-$ reductase N.S., hemoglobin N.S. In comparing the effect of 47 ppm $NO_3^-$-N vs. 104 ppm $NO_3^-$-N at 79 days the difference in nitrate reductase activity was significant at the 0.01 level. Other differences in nitrate reductase or hemoglobin obtained from comparing the 47 ppm $NO_3^-$-N and 47 ppm $NH_4^+$-N with the 104 ppm $NO_3^-$-N and 104 ppm $NH_4^+$-N treatments, respectively, were not significant.

TABLE V

EFFECT OF IRON LEVELS ON CHLOROPHYLL CONTENT AND DRY WEIGHT OF SOYBEAN PLANTS, AND HEMOGLOBIN CONTENT AND NITRATE REDUCTASE ACTIVITY OF SOYBEAN NODULES*

| LEVEL OF IRON (PPM) | CHLOROPHYLL CONTENT OF FRESH LEAVES (MG/G) | DRY WT OF PLANTS (G/CULTURE) | HEMOGLOBIN (μG/G FRESH NODULES) | NITRATE REDUCTASE ACTIVITY (mμMOLES $NO_2^-$/10 MIN/MG PROTEIN) |
|---|---|---|---|---|
| *Experiment 5A* | | | | |
| 0.005 | 0.4 ± 0.2 | 3.1 ± 0.2 | 43 ± 3 | 16 ± 7 |
| 0.015 | 0.5 ± 0.1 | 3.0 ± 0.5 | 41 ± 6 | 11 ± 7 |
| 1.00 | 1.7 ± 0.2 | 7.2 ± 1.5 | 116 ± 6 | 70 ± 6 |
| *Experiment 5B* | | | | |
| 0.005 | 0.3 ± 0.1 | 1.4 ± 0.1 | 35 ± 10 | 14 ± 11 |
| 0.10 | 0.8 ± 0.2 | 3.9 ± 0.3 | 156 ± 17 | 114 ± 27 |
| 1.00 | 2.1 ± 0.4 | 6.8 ± 0.7 | 227 ± 18 | 220 ± 64 |

* All results of determinations reported are means with standard deviations of four replications. For other details of the experiments see Materials and Methods.

culture vessels, polyethylene carboys for storage of water and nutrient solutions, and all other equipment used for growth of the plants were washed first with detergent and hot water, then with 4 *N* HCl, and finally rinsed with sufficient demineralized water until the rinse water attained a pH value of 5.2 or greater. Demineralized water was prepared by permitting distilled water to flow slowly through a column (35 cm by 8 cm) of anion and cation resins prepared by mixing two parts of hydroxyl saturated IRA400 with one part of hydrogen saturated IR120 (Rohm and Haas Company).

Stock solutions of the macro-elements for the nitrogen-free nutrient solution of Hoagland and Arnon (14) were purified for removing Mo by the CuS co-precipitation method (13, 19). $CaSO_4$ and $Ca(H_2PO_4)_2$ used in the nutrient solutions were prepared by mixing equivalent amounts of the purified solutions of $K_2SO_4$ and $CaCl_2$, and $CaCl_2$ and $KH_2PO_4$, respectively. The resulting precipitates were collected on Büchner funnels and then dried at 90° C to constant weight. Micro-nutrient elements were purified by recrystallization from demineralized water. Iron was supplied as the ferric chelate of ethylenediamine tetraacetate. Purified stock solutions of the necessary mineral salts were diluted to the desired concentration with the demineralized water, and the pH was adjusted to 6.5 with NaOH.

Four soybean seedlings, with cotyledons removed, were transferred to each of the polyethylene culture vessels filled with nutrient solution. All culture vessels were covered with polyethylene tops, each fitted with holes through which the seedlings were inserted into the nutrient solutions. Cultures were aerated with an air stream which was passed through water to remove solid impurities.

TABLE VI

EFFECT OF MOLYBDENUM LEVELS IN CULTURE SOLUTIONS ON NITROGEN CONTENT AND DRY WEIGHT OF SOYBEAN TOPS AND NITRATE REDUCTASE ACTIVITY AND HEMOGLOBIN CONTENT OF NODULES*

| LEVEL OF MO (PPM) | DRY WT OF TOPS (G) | TOTAL N IN TOPS (% DRY WT) | HEMOGLOBIN (μG/G FRESH NODULES) | NITRATE REDUCTASE ACTIVITY (mμMOLES $NO_2^-$/10 MIN/MG PROTEIN) |
|---|---|---|---|---|
| 0.00 | 13.1 | 1.75 | 110 | 105 |
| 0.005 | 14.9 | 2.78 | 160 | 250 |
| 0.050 | 14.8 | 2.93 | 141 | 180 |
| LSD** (0.05) | N.S. | 0.20 | 36 | 58 |
| (0.01) | | 0.29 | 51 | 82 |

* Values reported are means of six replications. All determinations in this table were carried out on plant material collected at the end of the experiment (72 days growth). After a period of 57 days of growth, samples of nodules were collected and assayed for nitrate reductase activity and the mean values in the same units used in this table were 208, 219, and 290 for the 0.0, 0.005 and 0.050 ppm molybdenum treatments, respectively. The LSD (0.05) for these determinations was 36. Differences in the hemoglobin content of nodules collected after 57 days were not significant. See Materials and Methods for other details of the experiment.

** Least significant difference.

The basic nutrient solution utilized was the nitrogen-free solution of Hoagland and Arnon (14) which was prepared from the purified salts. Concentrations of molybdenum in the solutions used for the various culture series are indicated in table VI. The nutrient solution in each container was changed at intervals of approximately ten days.

The experiment was arranged in a randomized block in the greenhouse; each treatment was applied to six replicate cultures. Because the experiment was conducted during November, December, and January, supplemental light was supplied as indicated in experiment 3. Plants were harvested after 72 days of growth; however, the tops of all plants were removed at a height of 6 inches above the vessel top after growth periods of 30 and 57 days, respectively, after planting. This procedure was necessary in order to accentuate deficiency symptoms.

Enzyme Assays and Other Determinations: For assays of nitrate reductase activity, nodules were removed from roots and placed immediately in cold distilled water. All preparatory operations were carried out at 0 to 4° C. Immediately after the nodules were harvested they were washed repeatedly in cold distilled water, blotted dry, and macerated with a mortar and pestle in 0.005 M K-phosphate buffer at pH 7.5 (4 ml per g of nodules). The resulting slurries were squeezed by hand through four layers of cheesecloth and then the liquid portions were centrifuged at 30,000 × G for 5 minutes. Sediments containing the bacterial cells were resuspended, with the aid of a Ten Broeck homogenizer, in a volume of 0.1 M K-phosphate buffer, pH 7.5, equal to the volume of buffer used for macerating the nodules. These suspensions were used for the nitrate reductase assays.

The complete assay mixture for nitrate reductase consisted of the following, expressed as micromoles: 10, K-phosphate buffer at pH 7.5; 10, $NaNO_3$; 10, Na succinate at pH 7.5; and sufficient water to adjust the final volume to 0.5 ml. The assay was initiated by adding 0.1 or 0.2 ml of the bacterial suspension. The procedure for nitrate reductase assays was that described by Evans and Nason (10), modified as by Evans (9). All assays were carried out aerobically at 30° C for 10 minutes unless otherwise stated. The red diazo-dye concentration was determined spectrophotometrically at 540 mμ after centrifuging (3500 × G for 20 min) the suspensions in polyethylene tubes (nitrocellulose tubes gave high blank readings). Assay mixtures containing nitrite instead of nitrate showed that nitrite was not reduced in the assay mixtures.

The supernatants obtained after centrifugation of the macerated nodules were used for determining the hemoglobin. The pigment was estimated by the pyridine-hemochromogen method, following a procedure which is essentially the same as that described by Keilen and Hartree (15). Standard pyridine hemochromogen solutions were prepared from recrystallized hemin (Nutritional Biochemicals Co.). Spectrophotometric determinations were made with a Beckman spectrophotometer (Model DU). Determinations of the hemoglobin contents of nodules from velvet bean plants (table III) were complicated by a black pigment which is present in the nodules of this species. Because samples of nodules collected after growth periods of 23 or 107 days in this experiment (table III) contained no detectable hemoglobin, the mean optical densities obtained from extracts of these nodules were used to correct determinations on other samples in this experiment. Hemoglobin determinations in this experiment, therefore, are approximations.

Dry weights were determined after the plant material was dried in a forced draft oven for 24 hours at 80° C. Total N content of the plant tissue was determined by the Kjeldahl method with selenium as catalyst. Protein content of the bacterial suspensions was estimated by determining total N and multiplying by the factor 6.25. Chlorophyll was determined on 1 g samples of leaf tissue by the procedure described by Arnon (2).

## Results

Distribution of Enzyme: Nodules from several leguminous species were tested for nitrate reductase activity with succinate as the electron donor (table I). From these data it is apparent that succinate-nitrate reductase activity is greatest in nodules from soybean plants, and this observation is in agreement with the results reported by Evans (9) who used DPNH as electron donor. Activities reported in table I may not be maximal for a given legume, because, as shown subsequently, activity of this enzyme varies with the plant age. It is interesting that nitrate reductase activity varied considerably within one cross-inoculation group. Peanut, cowpea, velvet bean, crotalaria, and lespedeza plants are considered (8) to be in the same inoculation group; however, as indicated by the data, the nitrate reductase activity of the Rhizobium cells from nodules of these different species varied widely. The enzyme has been identified not only in nodules of the species reported in table I but also has been detected qualitatively in extracts of nodules of *Sesbania exhaltata* and *Indigofera hirsuta*. No activity was observed in Alnus nodules.

Effect of Legume Age: The effect of age of soybean plants on nodule nitrate reductase activity is shown in table II. Hemoglobin contents of the nodules as well as the dry weights and total N contents of tops also are given, because Virtanen et al (20, 21) have demonstrated a close correlation between hemoglobin content of nodules and the quantity of $N_2$ fixed by the symbiotic system. The results reported in table II appear to confirm the observations of Virtanen et al (20, 21) that the rate of increase in $N_2$ fixation parallels the rate of increase of hemoglobin content of nodules. A similar relationship appears to exist between the nitrate reductase activities of Rhizobium

cells from nodules, and either the hemoglobin contents of nodules or the increases in nitrogen fixed by the plants.

The dry weights of velvet bean (*Stizolobium deeringianum*) plants and the hemoglobin contents and nitrate reductase activities of nodules from this species also were determined throughout the growth period (table III). Neither hemoglobin nor nitrate reductase activity of nodules could be detected when the first samples were collected after 23 days of growth. As indicated by analysis of nodules at 36, 43, and 64 day sampling periods, both the enzyme activities and hemoglobin contents of nodules increased sharply and maintained somewhat of a plateau during this period. After 10 days of growth, however, both components exhibited a sharp decline. Marked increases in dry weights are shown between the 64 and 107 day sampling periods indicating that maximum growth had not been obtained during the experiment.

Effect of Combined Nitrogen in Cultures: It is known generally that the addition of fixed N to either the asymbiotic or symbiotic nitrogen-fixing systems markedly reduces the rate of nitrogen fixation. Since it has been reported that nitrate reductase activity is correlated, in some unknown manner, with the nitrogen-fixing efficiency of strains of *R. japonicum* (5), studies were conducted to determine the effect of combined nitrogen on the activity of nitrate reductase. In table IV, the results of an experiment are recorded in which the effect of different levels of combined nitrogen on nitrate reductase activity, hemoglobin content of soybean nodules, and the dry weight of soybean tops has been investigated. Differences in the mean values of certain determinations in this experiment were statistically significant at a probability level of 0.05 or greater, and, as indicated in the footnote to table IV, differences in other comparisons were not significant. From the results shown in table IV, it appears that addition of nitrate to the growth medium, in comparison with nitrogen gas as the sole nitrogen source, significantly decreased the activity of the nodule nitrate reductase. Although there was not a significant difference between the low nitrate and high nitrate treatment at the 46 day sampling, there was a significant difference between these treatments at the 79 day sampling. The nitrate treatments, when compared with those lacking a source of combined nitrogen, also decreased the hemoglobin content of the nodules, but this difference was manifested only at the 46 day sampling date. At the 79 day sampling date, considerable numbers of nodules from plants receiving the various treatments had become senescent and showed evidence of breakdown of the hemoglobin pigment. This complication undoubtedly increased the variability among samples from replicate cultures.

A comparison of mean nitrate reductase activity of nodules from plants receiving all ammonium N treatments with that of nodules from plants receiving treatments lacking a source of combined N showed that the addition of ammonium N resulted in a significant reduction in activity at the 46 day sampling date. A significant difference, however, was not exhibited at the 79 day sampling date. The difference between the hemoglobin content of the nodules from cultures supplied with ammonium N and that of nodules grown without a source of combined nitrogen was not statistically significant at either sampling date.

Another experiment, (data not presented in tabular form) with the same objectives as that reported in table IV, was carried out for 70 days. One series of cultures was supplied with atmospheric nitrogen only. A second and third series received 47 and 94 ppm N, respectively, in the form of $Ca(NO_3)_2$, and a fourth and fifth series received 47 and 94 ppm N, respectively, in the form of $(NH_4)_2SO_4$. Single samples of nodules were collected from plants from each series after 28 days of growth. Duplicate cultures receiving each treatment were sampled at 46, 59, and 70 days after planting. Samples of nodules were assayed for nitrate reductase; the nodules exhibited a trend similar to that indicated by the data in table II. Enzyme activities on a protein basis were relatively low in all treatments at the first sampling date, reached a maximum at the 46 day sampling date, then declined at the 59 and 70 day sampling dates. The most striking effect of nitrogen treatment was exhibited after 46 days of growth. The mean specific activities of nodules from the treatments receiving 47 and 94 ppm N in the form of $Ca(NO_3)_2$ were 194 and 178, respectively, and the mean specific activities of nodules from plants receiving 47 and 94 ppm N in the form of $(NH_4)_2SO_4$ were 175 and 162, respectively. In comparison, the mean specific activity of the nodules from plants grown without a combined source of nitrogen was 233. At the 59 day sampling period, nodules from plants grown with $N_2$ exhibited greater enzyme activity than those from cultures receiving combined N. These results, therefore, generally confirm those reported in table IV, which indicate that adding combined nitrogen results in a decrease in activity of nodule-nitrate reductase. The effect is most pronounced at that period during plant growth when the greatest enzyme activity is exhibited.

Effects of Levels of Iron and Molybdenum in Nutrient Solutions: Iron or molybdenum have been implicated or demonstrated to be involved in electron transport from substrate to nitrate in all nitrate reductases reported to date (18). Kinetic studies on the inhibition of nodule nitrate reductase by cyanide have suggested that two or more cyanide sensitive sites are required for electron transport from either DPNH or succinate to nitrate (7). The effect of deficiencies of both Fe and Mo on nodulated soybean plants was studied, therefore, in an effort to determine whether or not levels of these elements in culture solutions would appreciably influence activity of the nodule enzyme. Results are presented in table V of two separate experiments in which the effect of different levels of Fe in culture solutions was studied

on chlorophyll contents of leaves, dry weights of plants, and the hemoglobin contents and nitrate reductase activities of nodules. The 0.005 or 0.015 ppm Fe levels in experiment 5A and the 0.005 and 0.10 ppm level in experiment 5B, when compared with the 1.0 ppm level in each of the experiments, resulted in a striking and consistent decrease in chlorophyll content of leaves, dry weight of plants, and hemoglobin content and nitrate reductase activity of nodules.

The influence of different levels of molybdenum in culture solutions on growth and nitrogen content of soybean tops and hemoglobin content and nitrate reductase activity of nodules is shown by the data in table VI. It was difficult in this experiment to obtain pronounced molybdenum deficiency symptoms. It was necessary to remove the upper vegetative portions of plants at two different dates during the growth period (see Materials and Methods).

Differences in the dry weight of plant tops were not influenced significantly by the culture treatment; however, as indicated by the data (table VI), the nitrogen content of plants grown with nutrient solutions lacking added molybdenum was clearly less than that of plants grown with solutions containing molybdenum. This difference and the chlorotic appearance of leaves of plants from cultures lacking molybdenum provided evidence of molybdenum deficiency. Both the hemoglobin content and nitrate reductase activity of nodules were strikingly reduced in the deficient cultures and reached a maximum in the cultures receiving the intermediate molybdenum treatment (0.005 ppm). When plants were harvested (after 72 days of growth), the hemoglobin content and nitrate reductase activity of nodules from cultures receiving the highest level of molybdenum were less than that in nodules from cultures receiving the intermediate level. As indicated in the footnote to table VI, samples of nodules also were collected from all cultures after 57 days of growth. The mean specific activities of the nodule nitrate reductase were 208, 219, and 290 for the 0.0, 0.005, and 0.05 ppm molybdenum treatments, respectively. In view of these values and the trend in activities of the nodule nitrate reductase during the growth season (table II), it seems quite probable that the decline in enzyme activity of nodules from the highest molybdenum treatment (table VI) may have been caused by a differential effect of treatment on the maturity of plants.

In another experiment (to be reported elsewhere) the addition of molybdenum to deficient soils used as a culture medium for soybeans resulted in a statistically significant increase in the nitrate reductase activity of nodules.

## Discussion

Experimental results reported in this manuscript provide evidence that the activity of the nitrate reductase in the nodules of soybean and velvet bean plants goes through a phasic change during the growth period. The specific activity of the enzyme in soybean nodules is relatively low during the early part of the growth season, reaches a maximum during the ninth week of growth, and then declines (table II). In the experiment with velvet bean plants (table III), maximum activity was observed during the fifth and sixth weeks of growth; relatively low activities were exhibited during the early parts and near the end of the growth period. The phasic change of nitrate reductase activity with increasing nodule age is similar to changes in respiratory activity of bacilli from nodules (3) except that a decrease of nitrate reductase activity is observed when the nodules approach senescence. This decrease in nitrate reductase activity is in contrast to an increase of respiratory activity of the nodule bacilli as the nodules become senescent (3). Although there was considerable variability among determinations of both hemoglobin contents and nitrate reductase activities of nodules from these species, it seems apparent that the trends of both these determinations were somewhat parallel. Virtanen et al (20) have discussed the phasic transfer of nitrogen from nodules to the host plant and have pointed out that the hemoglobin content of nodules parallels the increase in nitrogen content of leguminous plants during growth. It was postulated, therefore, that hemoglobin is involved in the nitrogen fixing process. The experiments of Hamilton et al (12) and Bergerson and Wilson (4), which indicate that hemoglobin is involved in electron transport in the symbiotic nitrogen fixing system, support Virtanen's postulation.

In view of the apparent correlation of nitrate reductase activities and hemoglobin contents of nodules during growth periods, and the fact that the nitrogen fixing efficiencies of different *Rhizobium japonicum* strains are correlated positively with nitrate reductase activities of nodules (5) it seems logical to postulate that the nodule nitrate reductase system is related in some manner to the nitrogen fixation process. It is possible that the decrease in nodule nitrate reductase activity near the end of the growth period of legumes is caused by a decreased permeability of the bacterial cells to nitrate. In this regard, evidence has been presented (11) indicating that cells of *E. coli* become increasingly impermeable to nitrate as they become older. If permeability were a factor responsible for the decline of nitrate reductase activity of nodule Rhizobia during the growth periods, then one would expect that the permeability barrier could be overcome by preparation of cell-free extracts. No evidence to date would support the contention that changes in cell permeability are responsible for the observed trends of nodule nitrate reductase activity during growth; however, the possibility of permeability being a factor has not been ruled out.

It was shown that the application of nitrate, and in some cases, ammonium nitrogen to soybean plants decreased the activity of nitrate reductase, but it is not known if this observation is related to the general observation that fixed nitrogen suppresses nitrogen-fixation. The decrease in enzyme activity caused by applications of nitrate, possibly may be explained by

an accumulation of toxic levels of nitrite from the nitrate reductase reaction. Some nitrate reductases (17), however, have been reported to be adaptive to nitrate and, therefore, inhibition of activity by the levels added to nutrient solutions was not expected. No analogous explanation can be offered for the observed decrease in nitrate reductase activity resulting from the application of ammonium nitrogen. One may postulate that the nitrate reductase is related in some manner to the nitrogen fixing process, and that the nitrate reductase reaction is not utilized when fixed nitrogen is available. It was reported by Wainwright and Nevill (22) that $(NH_4)_2SO_4$ exerted an inhibitory effect on formation of nitrate reductase in *E. coli*.

It is clear that both Fe and Mo deficiencies have a pronounced influence on the activity of nodule nitrate reductase when assayed with succinate as the electron donor. Whether or not both these metals are integral parts of the electron transport mechanism for nitrate reduction in *R. japonicum* is not known. It is of interest, however, that an Fe requirement has been shown for certain Rhizobium species when grown in pure culture on a medium (16) containing nitrate. Preliminary experiments in this laboratory with a pure culture of *R. japonicum* have shown that Mo not only is necessary for growth on a nitrate medium, but also is required for maximum nitrate reductase activity. Whether or not the nitrate reductase of nodules is the same enzyme as that present in pure cultures of Rhizobium is not known. The evidence, with DPNH as the electron donor, indicates that the specific activity of the enzyme in Rhizobia from nodules of plants grown without combined nitrogen is at least 8- to 10-fold greater than that of pure cultures of Rhizobium grown in media containing nitrate.

One might consider that nodule nitrate reductase plays its physiological role in the metabolism of traces of nitrate that seem to be inevitably present in all types of culture media. This view is difficult to accept for the following reasons. A. The specific activity of the nodule enzyme is greatest when legumes are grown in the absence of combined nitrogen, and the addition of nitrate or ammonium nitrogen to cultures results in reduced activity. This is in contrast to observations on other organisms which require nitrate as an inducing agent for nitrate reductase formation. B. Nodule nitrate reductase activity seems to be correlated with hemoglobin content of nodules and the increase in nitrogen content of legumes during the growth season. C. The activity of the enzyme in nodules of soybean plants inoculated with different strains of *R. japonicum* is positively correlated with the nitrogen fixing efficiencies of the various strains.

There is no evidence to support the view that nitrate is an intermediate in the nitrogen fixing process. In fact, experiments with isotopic nitrogen (to be published elsewhere) have shown that excised soybean nodules or root systems with attached nodules rapidly fix $N^{15}$, yet, during periods ranging from ½ to 16 hours after exposure, the isotope fails to equilibrate with the traces of nitrate that are found in and isolated from the roots or nodules. It is unnecessary to assume that nitrate is the natural oxidant for the nodule enzyme, since it is well established that enzymes such as aldehyde dehydrogenase, xanthine oxidase, and certain hydrogenases will function with nitrate as the oxidant under certain conditions.

Despite the apparent correlations shown here and elsewhere (5) of nitrate reductase activity and $N_2$-fixing potential of nodules, the nitrate reducing system of nodules should be regarded (7) as a system involved in nitrate respiration until evidence is presented to demonstrate directly its role, if any, in $N_2$-fixation.

## Summary

The nitrate reductase activity of Rhizobium cells from nodules was studied in relation to factors known to influence $N_2$-fixation by nodules. A definite pattern of enzyme activity, as affected by age of plant, was found. It consisted of an increase of the specific activity of nitrate reductase until an optimum was reached and then a decline of activity when the nodules approached senescence. The change of hemoglobin content of the nodules from soybean and velvet bean plants followed, in general, a similar pattern.

The application of nitrate-N, and in some cases, ammonium-N to soybean plants resulted in a decreased activity of nitrate reductase in nodules. The hemoglobin content of nodules also was decreased by the nitrate treatments but not by the ammonium treatments.

Both Fe and Mo deficiencies resulted in a pronounced reduction in the activity of nodule nitrate reductase and the content of hemoglobin in nodules.

The evidence for a possible role of nitrate reductase in $N_2$-fixation is discussed.

## Literature Cited

1. Anderson, I. C. and H. J. Evans. 1956. Effect of manganese and certain other metal cations on isocitric dehydrogenase and malic enzyme activities in *Phaseolus vulgaris*. Plant Physiol. 31: 22–28.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. Bergerson, F. J. 1958. The bacterial component of soybean root nodules; changes in respiratory activity, cell dry weight, and nucleic acid content with increasing nodule age. Jour. Gen. Microbiol. 19: 312–323.
4. Bergerson, F. J. and P. W. Wilson. 1959. Spectrophotometric studies of the effects of nitrogen on soybean nodule extracts. Proc. Nat. Acad. Sci., U. S. 45: 1641–1646.
5. Cheniae, G. M. and H. J. Evans. 1957. On the relation between nitrogen fixation and nodule nitrate reductase of soybean root nodules. Biochim. Biophys. Acta 26: 654–655.

6. CHENIAE, G. M. and H. J. EVANS. 1958. Influence of quinones on electron transport systems from Rhizobia. Fed. Proc. 17: 201.
7. CHENIAE, G. M. and H. J. EVANS. 1959. Properties of a particulate nitrate reductase from the nodules of the soybean plant. Biochim. Biophys. Acta 35: 140–153.
8. ERDMAN, T. W. 1948. Legume inoculation: What it is; What it does. U. S. Dept. Agr. Farmer's Bulletin No. 2003, U. S. Government Printing Office, Washington.
9. EVANS, H. J. 1954. Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiol. 29: 298–301.
10. EVANS, H. J. and A. NASON. 1952. The effect of reduced triphosphopyridine nucleotide on nitrate reduction by purified nitrate reductase. Arch. Biochem. Biophys. 39: 234–235.
11. FORKAS-HIMSLEY, H. and M. ARTMAN. 1957. Studies of nitrate reduction by *Escherichia coli*. Jour. Bacteriol. 74: 690–692.
12. HAMILTON, P. B., A. L. SHUG, and P. W. WILSON. 1957. Spectrophotometric examination of hydrogenase and nitrogenase in soybean nodules and Azotobacter. Proc. Nat. Acad. Sci., U. S. 43: 297–304.
13. HEWITT, E J. and E W. JONES. 1949. Molybdenum as a plant nutrient. II. Effect of molybdenum deficiency on some Brassica crops. Pp. 58–63. Bristol University. Agricultural and Horticultural Research Station Report.
14. HOAGLAND, D. R. and D. I. ARNON. 1950. The water-culture method for growing plants without soil. Cal. Agr. Expt. Sta. Circular No. 347, University of California, Berkeley. (Revised by D. I. Arnon from 1938 edition.)
15. KEILEN, D. and E. F. HARTREE. 1951. Purification of horse-radish peroxidase and comparison of its properties with those of catalase and methaemoglobin. Biochem. Jour. 49: 88–104.
16. LILLY, V. G. and L. H. LEONIAN. 1945. The interrelationship of iron and certain accessory factors in the growth of *Rhizobium trifolli*, Strain 205. Jour. Bacteriol. 50: 383–394.
17. NASON, A. and H. J. EVANS. 1953. Triphosphopyridine nucleotide-nitrate reductase in Neurospora. Jour. Biol. Chem. 202: 655–673.
18. NASON, A. and H. TAKAHASHI. 1958. Inorganic nitrogen metabolism. Ann. Rev. Microbiol., C. E. Clifton, ed. 12: 203–246. Annual Reviews, Inc., Palo Alto, Cal.
19. STOUT, P. R. and W. R. MEAGHER. 1948. Studies of molybdenum nutrition with radioactive molybdenum. Science 108: 471.
20. VIRTANEN, A. I., J. ERKAMA, and H. LINKOLA. 1947. On the relation between nitrogen fixation and leghaemoglobin content of leguminous root nodules. II. Acta Chem. Scand. 1: 861–870.
21. VIRTANEN, A. I., J. JORMA, H. LINKOLA, and A. LINNASALINI. 1947. On the relation between nitrogen fixation and leghaemoglobin content of leguminous root nodules. Acta Chem. Scand. 1: 90–111.
22. WAINWRIGHT, S. D. and A. NEVILL. 1956. The influence of depletion of nitrogenous reserves upon the phenomenon of induced enzyme biosynthesis in cells of *Escherichia coli*. Jour. Gen. Microbiol. 14: 47–56.

# ISOCITRITASE, GLYCINE-ALANINE TRANSAMINASE, AND DEVELOPMENT IN BLASTOCLADIELLA EMERSONII [1, 2, 3]

HOWARD D. McCURDY, JR.,[4] AND EDWARD C. CANTINO

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY,
MICHIGAN STATE UNIVERSITY, EAST LANSING

The discovery (9) that the development of a new aquatic fungus, *Blastocladiella emersonii*, along either of two distinct morphogenetic pathways leading to ordinary colorless (OC) or resistant sporangial (RS) plants could be determined by a simple manipulation of the external environment, presented an excellent opportunity for studying the relationship between metabolism and morphogenesis. Even with this very simple, two-celled organism, however, almost a model system in fact, the relationship is a complex one, and the progress of our understanding inevitably slow. The comparatively recent finding (14) that light plays a role in development has added an interesting but complicating aspect to the story.

Studies during the past decade have led us (11, 13, 18, 58) to postulate mechanisms for the morphogenetic and light phenomena in Blastocladiella. An integral part of these is the key role of an enzyme, isocitritase; its presence was implied but not explicitly demonstrated. In the work reported here, the presence of isocitritase in Blastocladiella has been confirmed, the enzyme partially purified, and its properties studied. A second enzyme, glycine-alanine transaminase, presumably involved in the in vivo role of isocitritase, has been similarly examined. After the development of new culture techniques, the relationship of these enzymes to differentiation was investigated in detail.

## MATERIALS AND METHODS

*B. emersonii* was grown on Difco CANTINO PYG AGAR and BROTH media. Resistant sporangial (RS) plants were cultivated in PYG broth containing 0.75 g $NaHCO_3$/liter (38). For partial purification of enzymes, 1.5 l of PYG were inoculated with heavy spore suspensions, continuously aerated, and harvested just before the population produced one generation of plants. The latter were washed with 2 l of water and sucked dry. Additional details are found in the text.

Biochemicals were obtained as follows: amino, ketoglutaric, and oxalacetic acids from Nutritional Biochemical Corp.; triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), sodium glyoxylate monohydrate, L-isocitric acid lactone (allo free), and pyridoxal-5-phosphate, from Sigma Chemical Co.; pyruvic acid from California Corp. for Biochemical Research.

Isocitric acid was prepared from its lactone by hydrolysis with KOH; the hydrolysate contained 50 % D-isocitric acid (by assay with pig heart isocitric dehydrogenase (43). Sodium pyruvate was prepared from the acid (46) and recrystallized from a water-alcohol solution. Inorganic chemicals and organic solvents were reagent grade. Glass beads (No. 100) used for grinding plant tissue in the Servall Omnimixer were from the Minnesota Mining and Manufacturing Co.

SPECIFIC METHODS FOR STUDIES OF ISOCITRITASE. Glyoxylic acid was assayed as its 2,4-dinitrophenylhydrazone (DNPH) colorimetrically (25), and spectrophotometrically by an adaptation of the method of Smith and Gunsalus (55). Keto acids were chromatographed as DNPH derivatives according to Cavallini (19) in butanol-ethanol-water (BEW; 4:1:5), butanol-acetic acid-water (BAW; 4:1:5), 1-propanol-ammonia-water (PAW; 6:3:1), and two-dimensionally in phenol-water (PW; 2.5:1). Protein was determined turbidimetrically with trichloroacetic acid (TCA) according to Stadtman et al (57). Estimates of protein concentration on purified enzyme preparations were made at 215 and 225 mμ (60). Succinic acid was determined manometrically with succinoxidase from pig heart according to Krebs as described by Umbreit et al (59). Isocitric acid was determined with pig heart isocitric dehydrogenase (43).

Isocitritase activity was determined at 30° C in 3.1 ml reaction mixtures containing, in μM: D,L-isocitrate, 40; cysteine · HCl, 5; $MgCl_2$, 10; phosphate, pH 7.4, 200. The reaction was initiated by adding substrate and stopped by adding 0.3 ml *2 N* HCl. When necessary, protein was precipitated with 0.02 ml 10 % $Na_2WO_4 \cdot 2H_2O$ and removed by centrifuging. After diluting to 10 ml, 1 ml of 0.1 % dinitrophenylhydrazine in *2 N* HCl was added to 1 ml of reaction mixture. After 6 minutes at room temperature, 2 ml 95 % ethyl alcohol was added, followed by

[1] Received for publication October 27, 1959.

[2] This work was supported by research grants to the second author from the National Institutes of Health and the National Science Foundation.

[3] Paper No. 59–22 from the Department of Botany and Plant Pathology, Michigan State University, East Lansing.

[4] Present address: Department of Biology, Essex College, Assumption University of Windsor, Windsor, Canada.

4 ml of 1.5 *N* NaOH. After 3.5 minutes, the color was read at 490 and 540 mμ. Under our conditions, the optical density (O.D.) ratio (490 mμ: 540 mμ) of the DNPH derivatives of glyoxylate, pyruvate, and ketoglutarate were about 1.5, 1.0, and 0.8 respectively; thus, the O.D. ratio revealed the presence of keto acids other than glyoxylate, and derivation of simultaneous equations permitted calculation of the individual concentrations of the acids present. One unit of isocitritase activity was defined as the amount of enzyme catalyzing formation of 1 μM glyoxylate/10 minutes; and specific activity, the number of units/mg protein.

Specific Methods for Studies of Glycine-alanine Transaminase. Glycine and alanine were determined quantitatively (31), following their separation with PAW on acid-washed Whatman No. 1 paper. For qualitative studies, amino acids were resolved with BEW, PAW, and BAW, and detected with 0.1 % ninhydrin in 95 % ethyl alcohol. Pyruvic acid was determined according to Straub, as outlined by Green et al (*27*), with slight modification. Mixtures of glyoxylate and pyruvate were assayed as the DNPH derivatives (as described previously). Glycine-alanine transaminase was determined at 37° C in 1 ml assay mixtures containing, in μM: sodium glyoxylate monohydrate, 10; L-alanine, 10: phosphate, pH 8.0, 40; $MgCl_2$, 5; and pyridoxal-5-phosphate, 10 μg. After 10 minutes the reaction was stopped with Straub test Reagents (a control is necessary to correct for a small amount of non-enzymatic transamination which occurs between glyoxylate and alanine in the strong alkali used in the test). A unit of transaminase was defined as the amount of enzyme catalyzing production of 1 μM pyruvate/10 minutes; specific activity, the number of units/mg protein.

Specific Methods for Studies of Physiology of Development. Uniform spore inocula, with known total dry weight and viable counts, were standardized turbidimetrically. Large Petri plates containing 40 ml PYG agar were inoculated with about $10^5$ viable spores; the latter were spread evenly over the surface and incubated at 27° C. After germination of the mature plants had begun, plates were flooded with 10 to 15 ml of sterile water. After about 30 minutes, a spore suspension was generally obtained containing from $10^6$ to $10^7$ spores/ml.

After a density determination, the suspension was centrifuged at 400 × G for exactly 1.5 minutes in 40 ml conical tubes, and the supernatant removed by pipette, care being taken not to disturb the loosely packed sediment. The spores were resuspended in the small amount of residual medium, transferred to 12 ml centrifuge tubes, and cooled to 10° C. The suspension was centrifuged at 1,600 × G for exactly 30 seconds, the supernatant decanted, and the sediment resuspended in $10^{-2}$ M phosphate, pH 7.0. Finally, the resuspended spores were washed by centrifugation as before, the supernatant decanted, and the spores resuspended again.

Careful adherence to this procedure was essential; when followed carefully, it resulted in zoospore suspension exhibiting 80 to 100 % mobility. Any deviation, however, generally led to the fragile zoospores rounding up and losing their flagella. Repeated centrifugation in distilled water or hypertonic solutions ($10^{-1}$ M), or high-speed centrifugation, generally led to loss of motility, deformation, and sometimes complete disintegration.

For preparation of homogenates, swarmers were centrifuged at 1,600 × G for 3 minutes. The pellet was resuspended in five volumes of phosphate ($10^{-1}$ M, pH 7.4) containing $10^{-4}$ M versene plus $10^{-3}$ M cysteine · HCl) and homogenized in a 3 ml glass homogenizer or ground with a carborundum-glass mixture in a mortar and pestle.

## Results

Isocitritase. Incubation of crude homogenates of Blastocladiella with isocitric acid led to the identification of glyoxylic acid as the product of an enzymatic reaction. However, ketoglutarate and pyruvate were always produced as well when long incubation periods were used; this was later shown to be due to enzymatic and chemical transaminations between glyoxylate and endogenous amino acids. The demonstration of the stoichiometry and properties of the isocitritase reaction were therefore dependent upon preliminary purification. In all cases where attempts were made to obtain active, particle-free preparations by means of high speed (22,000 × G) centrifugation of homogenates (prepared with glass homogenizer) most of the activity was always retained in the sediment. However, soluble preparations from OC plants were finally obtained from extracts of acetone-dried powders and extracts obtained by homogenization in a Servall Ommimixer.

Preparation of Isocitritase from Acetone Powders with Glass Homogenizer. A 5-day culture was harvested and homogenized in an ice bath for 10 minutes, (5.0 ml 2 × $10^{-2}$ M phosphate/gm wet wt). The homogenate was centrifuged at 22,000 × G, the supernatant discarded, and the sediment resuspended in the same volume of buffer. The centrifugation was repeated, the sediment resuspended in one-fifth volume of buffer, the latter treated with 10 volumes of acetone (−10° C), allowed to stand 10 minutes, filtered, washed, with two volumes acetone, and sucked dry. The mat was air dried, ground to a powder, and stored at −18° C in a vacuum desiccator.

Active extracts were prepared as follows: 1 g acetone-dried powder was stirred vigorously with 20 ml $10^{-1}$ M phosphate, pH 7.4, containing $10^{-3}$ M cysteine for 15 minutes, centrifuged at 22,000 × G, and the sediment discarded. To 10 ml of supernatant, which contained the enzyme, 3.2 g $(NH_4)_2SO_4$ was added in the cold, and the precipitate centrifuged off and resuspended in one-fourth the original volume of buffer.

PARTIAL PURIFICATION OF ISOCITRITASE FROM OMNIMIXER HOMOGENATES. Results of a typical fractionation are shown in table I.

*Step* 1: *Preparation of the crude extract.* Plants from 14 hour cultures were homogenized in $10^{-1}$ M phosphate, pH 7.4, containing $10^{-3}$ M cysteine and $10^{-4}$ M versene (5 ml buffer/g wet wt) with glass beads in the Omnimixer at 15,000 rpm for 3 minutes at −5° C. The homogenate was centrifuged in the cold at 22,000 × G and the supernatant used for the subsequent fractionation.

*Step* 2: *First* $(NH_4)_2SO_4$ *Fractionation (fraction A).* Twenty-two g $(NH_4)_2SO_4$ was added to 100 ml supernatant and the precipitate removed by centrifugation and discarded. To the supernatant was added 10 g $(NH_4)_2SO_4$/100 ml, the precipitate recovered by centrifugation, and redissolved in a fourth the original volume of $2 \times 10^{-2}$ M phosphate, pH 6.0.

*Step* 3: *Protamine sulfate treatment* (cf. Colowick and Kaplan (20)). Protamine sulfate (20 mg/ml $2 \times 10^{-2}$ M phosphate, pH 6.0) was added to the solution from step 2 such as to yield 0.27 mg protamine/mg protein. The precipitate was removed by centrifugation.

*Step* 4: *Second* $(NH_4)_2SO_4$ *Fractionation (fraction B).* The protamine sulfate supernatant was brought to 27 % saturation with $(NH_4)_2SO_4$, centrifuged, and the inactive sediment discarded. An additional 5.4 g/100 ml of original volume was introduced, the precipitate collected by centrifugation, and redissolved in one-fifth volume of $2 \times 10^{-2}$ M phosphate, pH 5.6.

*Step* 5: *Adsorption of impurities with* $Ca_3(PO_4)_2$ gel (cf. Keilin and Hartree (32). Sufficient gel was added to give a gel/protein ratio of one, the suspension equilibrated by stirring, and the gel removed by centrifugation and discarded.

*Step* 6: *Third* $(NH_4)_2SO_4$ *Fractionation (fraction C).* To 10 ml of supernatant was added 6.4 ml saturated $(NH_4)_2SO_4$, pH 7.5, the solution centrifuged, and the sediment discarded. An additional 3.1 ml of saturated $(NH_4)_2SO_4$ per 10 ml was added, the sediment recovered, and redissolved in $10^{-4}$ M phosphate, pH 7.4, containing $10^{-4}$ M cysteine and $10^{-4}$ M versene.

IDENTIFICATION OF GLYOXYLATE. Glyoxylate was identified as a product of the enzymatic reaction by chromatography of its DNPH in four different solvents, and by 2-dimensional co-chromatography in PW and BPW. It was further identified by means of the absorption spectrum of its DNPH (max., 455 mμ) and the alkali-instability of the latter; thus it was readily distinguishable from the DNPH of pyruvate and ketoglutarate, neither of which exhibits marked alkali instability.

STOICHIOMETRY AND REVERSIBILITY OF ISOCITRITASE REACTION. Stoichiometry was established with $(NH_4)_2SO_4$ preparations from acetone extracts and $(NH_4)_2SO_4$ fraction A. The results (table II) are in agreement with the established reaction:

$$\text{D-isocitrate} \rightleftharpoons \text{Glyoxylate} + \text{succinate}$$

The reversibility of the reaction was confirmed by incubating 20 μM of glyoxylate and succinate with the enzyme; 2.5 μM isocitrate/hour was produced.

PROPERTIES OF ISOCITRITASE. *Stability.* Frozen mats and acetone powders of Blastocladiella may be stored for several weeks without appreciable loss of activity, but crude extracts stored at 4° C may lose as much as 30 to 40 % of their activity in 4 hours. Partially purified enzyme solutions will also lose about 50 % activity on overnight storage at 4° C, but they may be stored for some weeks at −18° C with little loss.

*Reaction rate and effect of pH.* The rate of glyoxylate formation is directly proportional to en-

TABLE I

PARTIAL PURIFICATION OF ISOCITRITASE*

| STEP | FRACTION** | PROTEIN (MG/ML) | SPECIFIC ACTIVITY | % RECOVERY*** | PURIFICATION (-FOLD) |
|---|---|---|---|---|---|
| 1 | Crude extract | 4.1 | 0.31 | 100 | 1 |
| 2 | $(NH_4)_2SO_4$ Fraction A | 7.6 | 0.60 | 98 | 2 |
| 3 | Protamine Supernatant | 2.4 | 1.70 | 91 | 5 |
| 4 | $(NH_4)_2SO_4$ Fraction B | 3.0 | 4.00 | 55 | 13 |
| 5 | Gel Supernatant | 1.0 | 8.30 | 44 | 27 |
| 6 | $(NH_4)_2SO_4$ Fraction C | 0.8 | 16.20 | 26 | 52 |

* Reaction mixture: 5 μM cysteine. HCl; 10 μM $MgCl_2$; 200 μM phosphate, pH 7.4; 40 μM D,L-isocitrate; about one unit isocitritase; in 3.1 ml.; 30° C.

** All steps carried out at 0 to 2° C.

*** (Total units/total number units in original extract) × 100.

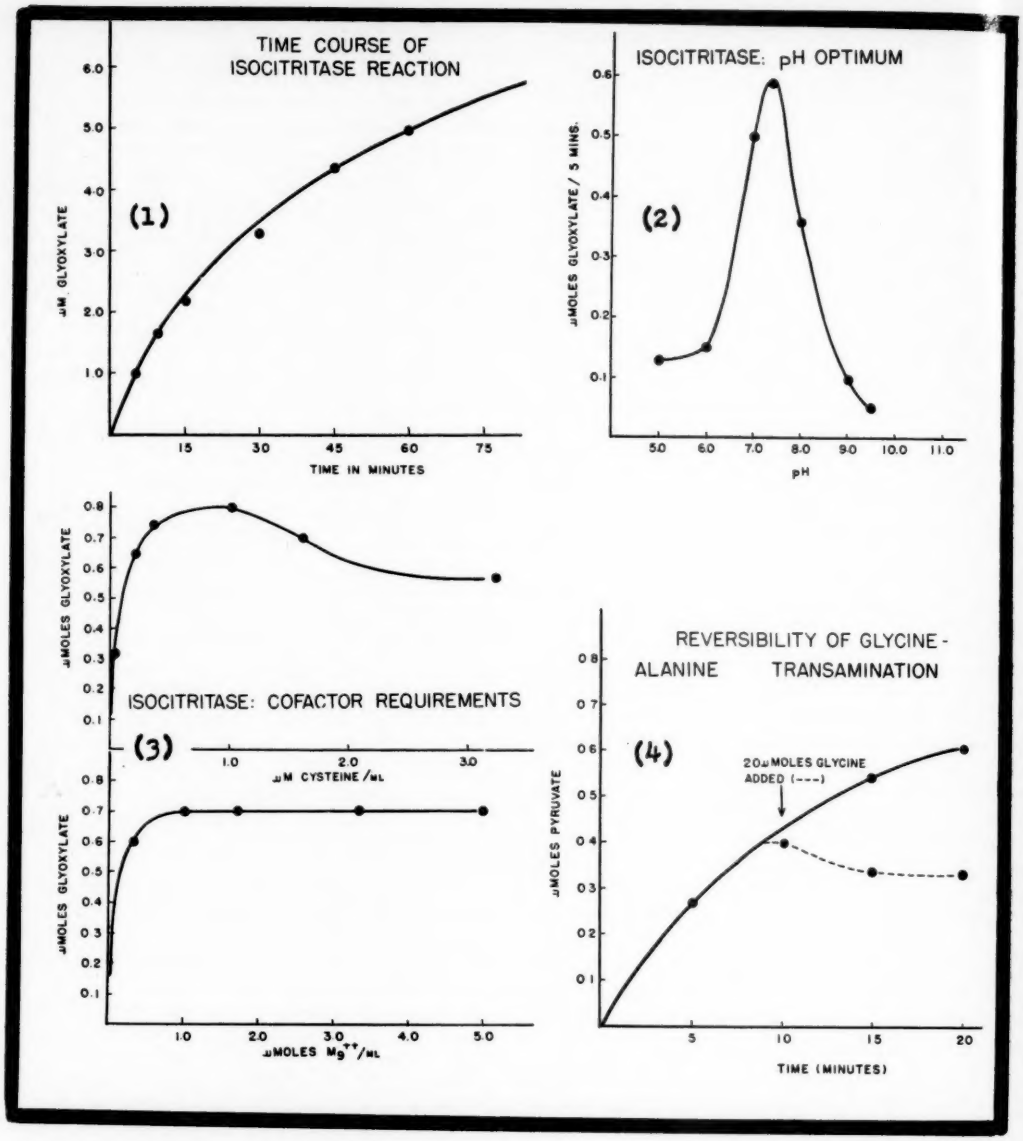



FIG. 1. Time course of the isocitritase reaction. Conditions as described for isocitritase assay (table I), but with incubation times as indicated.

FIG. 2. Isocitritase pH optimum. Conditions as for isocitritase assay (table I) except that a series of 5 minute incubations was used at the indicated pH. Enzyme: $(NH_4)_2SO_4$ fraction C.

FIG. 3. Isocitritase co-factor requirements. Conditions as for isocitritase assay (table I). Enzyme: $(NH_4)_2SO_4$ fraction C.

FIG. 4. Reversibility of glycine-alanine transaminase. Two parallel series of four reaction tubes were set up using the conditions described for the transaminase (tableIII). The reaction was initiated by addition of substrates and a tube from each set stopped at 5 minute intervals up to 20 minutes. As shown in one set, 20 μM glycine in 0.1 ml was added to the 15 and 20 minute tubes. Enzyme: Gel Supernatant B.

TABLE II

ISOCITRITASE STOICHIOMETRY*

| ENZYME | D-ISOCITRATE μM | GLYOXYLATE μM | SUCCINATE μM |
|---|---|---|---|
| $(NH_4)_2SO_4$-acetone fraction | | | |
| Expt. 1 | 4.9 | 5.0 | 5.4 |
| Expt. 2 | 4.0 | 5.3 | 4.1 |
| $(NH_4)_2SO_4$-fraction A | | | |
| Expt. 1 | 3.8 | 4.0 | ... |
| Expt. 2 | 5.2 | 4.9 | ... |

* Reaction mixture as in table I, but 1 hour incubation; enzyme, about two units.

zyme concentration up to about two units. The time course of the reaction is shown in figure 1. The enzyme exhibits a sharp pH optimum at 7.4 (fig 2); thus, assays were carried out at this pH and with about one unit of enzyme.

*Specificity.* Though neither pure D-isocitrate nor L-isocitrate was available, specificity for the D-isomer is implied by the stoichiometry data; that is, while the isocitric dehydrogenase used for determination of isocitrate is specific for the D-isomer, both isomers were always present in the isocitritase reaction mixture in equal concentrations.

Crude extracts did not produce DNPH-forming compounds when incubated with citrate or cis-aconitate. This is of particular interest since various workers (e.g. 21, 56, 61) have reported an enzyme from bacteria (citritase) which cleaves citrate to oxaloacetate and acetate. In particular, oxaloacetate was never detected as a product of isocitrate utilization by crude extracts or acetone powders of OC plants, though conditions were such that it would have been detected, and the presence of aconitase activity in such preparations has been demonstrated (17). Neither did they produce oxaloacetate from citrate as judged by measurements at 280 mμ (28).

COFACTOR REQUIREMENTS. Isocitritase requires a metallic ion for maximum activity as well as a sulfhydryl group. Glutathione was only 60 % as effective as cysteine. Though Mg was routinely employed as cofactor, Mn, at the same concentration, was equally effective (when tried with tris buffer systems). Cofactor saturation curves for both Mg and cysteine are shown in figure 3; both gave maximum activity at ca. $10^{-3}$ M.

EFFECT OF SUBSTRATE CONCENTRATION, TEMPERATURE, AND LIGHT. When isocitrate concentration (as the D-isomer) was plotted against reaction velocity, a typical substrate concentration curve was obtained; rearrangement of the data according to Lineweaver and Burk (37) permitted estimation of the Michaelis constant as $4.8 \times 10^{-4}$ M. Determination of reaction rates at several temperatures allowed graphical estimation of the activation energy according to the Arrhenius equation (42, 2) as E = 10, 700 calories/mole. The $Q_{10}$ of the reaction was 1.8. Finally, it was established that glyoxylate production in white light (300 ft-c.) and dark was identical.

GLYCINE-ALANINE TRANSAMINASE. During the early studies on isocitritase using crude preparations and long incubation periods, pyruvate was a frequent product of side reactions involving glyoxylate. Because it seemed likely that transamination between glyoxylate and endogenous amino acids was responsible, glyoxylate was incubated with a number of amino acids (L-glutamate, L-glutamine, L-aspartate, L-alanine, L-tyrosine, L-lysine, L-proline, L-leucine, L-histidine, L-arginine, and L-threonine) in the presence of boiled and unboiled crude enzyme and appropriate controls. Enzymatic transamination occurred only in those reaction mixtures containing L-alanine. Since it seemed likely that an alanine-glyoxylate transamination might constitute an important pulling reaction for removal of glyoxylate produced by isocitritase, further studies were carried out.

PARTIAL PURIFICATION OF GLYCINE-ALANINE TRANSAMINASE (see table III).

TABLE III

RESULTS OF TRANSAMINASE PURIFICATION*

| FRACTION | PROTEIN (MG/ML) | SPECIFIC ACTIVITY | % RECOVERY** | PURIFICATION |
|---|---|---|---|---|
| 1. Crude extract | 5.3 | 0.68 | 100 | 1 |
| 2. Protamine sulfate | 2.7 | 1.40 | 103 | 2 |
| 3. Acetone I | 2.2 | 11.00 | 96 | 16 |
| 4. Gel supernate B | 0.7 | 29.0 | 100 | 43 |
| 5. Acetone II | 0.9 | 55.0 | 60 | 80 |

* Reaction mixture: 10 μM sodium glyoxylate; 10 μM L-alanine; 40 μM phosphate, pH 8.0; 5 μM $MgCl_2$; 10 μg pyridoxal-5-phosphate; enzyme, under 0.7 units; in 1 ml, 37° C. Reaction stopped at 10 minutes with Straub reagents.

** (Total units/total No. units in original extract) × 100

TABLE IV

STOICHIOMETRY OF GLYOXYLATE-ALANINE TRANSAMINATION*

| TIME, HR | μM | | | GLYOXYLATE |
|---|---|---|---|---|
| | GLYCINE | PYRUVATE | ALANINE | |
| 1 | 2.9 | 2.7** | 2.5 | 2.8 |
| 2 | 3.5 | 3.6** | 3.3 | 3.2 |

* Reaction mixture as in Table III, but incubation times as indicated; reaction stopped with 0.09 ml. 2 *N* HCl; enzyme: Gel supernatant B.

** Determined by calculation from O.D. at 490 and 540 mμ, and Straub test. (averaged).

*Step* 1: *Preparation of crude extract.* Plants from 14 hour cultures were homogenized in water (4 ml/g) containing $10^{-4}$ M versene and glass beads (4 g/g plants) in an Omnimixer (16,000 rpm; −5° C) for 3 minutes. The homogenate was centrifuged at 22,000 × G, and the supernatant used for fractionation. Endogenous amino acids in the crude extract prevented direct estimation of initial transaminase activity. Therefore, the extract was dialyzed 20 hours against $2 \times 10^{-1}$ M phosphate, pH 7.4, at 4° C, after which it was assayed for activity. Since dialysis lowered the protein concentration through apparent denaturation of inactive protein, the initial, specific activity was expressed as μM pyruvate produced by the dialyzed preparation/mg protein in the original undialyzed material (prolonged dialysis of enzyme does not affect transaminase activity).

*Step* 2: *Protamine sulfate treatment.* Three-fourths ml of protamine sulfate (20 mg/ml) was added for every 100 mg crude extract protein at 0° C, and the precipitate removed by centrifugation at 30,000 × G.

*Step* 3: *First acetone fractionation.* Acetone (−10° C) was added until its concentration reached 47 % and the precipitate was removed by centrifugation and discarded. The active fraction was obtained by bringing the acetone concentration to 57 % at −10° C, the solution centrifuged, and the sediment resuspended in one-fifth the original volume of water. The solution was dialyzed 20 hours at 1° C against three changes of 100 volumes of water containing $10^{-4}$ M versene.

*Step* 4: *Negative adsorption with* $Ca_3(PO_4)_2$ gel. Enough gel solution (18.6 mg/ml) was added to the dialyzed fraction at 0° C to give a gel-protein ratio of 2:1. After adsorption equilibration (15 min), the gel was removed by centrifugation and discarded. The process was repeated once, and the supernatant used in the next step.

*Step* 5: *Final acetone fractionation.* To further concentrate the transaminase, enough acetone (−10° C) was added to give a concentration of 62 % at −5 to 0° C. The precipitate was recovered by centrifugation, resuspended in one-fourth volume water, and dialyzed 20 hours against three changes of 500 volumes of water.

STOICHIOMETRY, REVERSIBILITY, AND IDENTIFICATION OF PRODUCTS. Pyruvate was established as the product of the transamination between glyoxylate and alanine by paper chromatography of its DNPH in BEW, and by Straub's quantitative salicylaldehyde reaction. Glycine was identified by paper chromatography in PA, BEW, and BAW. Determinations of reactants and products after incubations of glyoxylate and alanine with enzyme (table IV) defined the reaction as:

Glyoxylate + L-alanine ⇄ glycine + pyruvate

To demonstrate reversibility, pyruvate and glycine were incubated in the usual reaction mixture. No disappearance of pyruvate was detected nor was alanine produced, in spite of the fact that the forward reaction had proceeded to only one-third conversion. Thus, it seemed likely that pyruvate or glycine was inhibitory. With this in mind, modified experiments were set up which did demonstrate the reversibility of the reaction (fig 4); glycine brought about apparent reversal. This was confirmed in another experiment in which 2.0 μM pyruvate and 20 μM glycine were incubated for 1 hour in the usual reaction mixture; this led to disappearance of 0.28 μM pyruvate and appearance of a corresponding amount of alanine.

PROPERTIES OF TRANSAMINASE. *Stability, effect of pH, and reaction rate.* The enzyme may be stored for at least a week at 4° C, or 2 months at −18° C, without loss of activity; it is also stable to prolonged dialysis against various buffers. Highest activity is obtained at pH 8.5. However, since considerable non-enzymatic transamination occurs here (fig 5), routine assays were carried out at pH 8.0. The amount of pyruvate produced was directly proportional to the amount of enzyme up to 0.7 units. The reaction proceeds to ca. one-third conversion (fig 6).

*Activators and inhibitors.* Neither prolonged dialysis against water, phosphate, nor 50 % saturated $(NH_4)_2SO_4$ caused the enzyme to exhibit stimulation by addition of pyridoxal-5-phosphate. Precipitation of the enzyme by dialysis against saturated $(NH_4)_2SO_4$ followed by dialysis against $2 \times 10^{-2}$ M acetate, pH 4.0 (cf. 64) caused complete, irreversible loss of activity which could not be restored by prolonged incubation with pyridoxal phosphate and Fe (+3), Mg, Cu (+2), etc. (41). In common with other pyridoxal phosphate enzymes, glycine-alanine transaminase was inhibited (table V) by KCN and hydroxylamine (20); the latter was overcome by $10^{-3}$ M pyridoxal phosphate. Isoniazid (22, 64), reportedly an inhibitor of vitamine $B_6$ enzymes, did not inhibit even at $10^{-2}$ M. So far, requirements for a metal ion have not been demonstrated for any transaminase; our glycine-alanine transaminase was no exception. Dialysis against water and various buffers containing versene failed to reveal the need for the metallic cofactor.

*Substrate specificity.* Incubations were carried

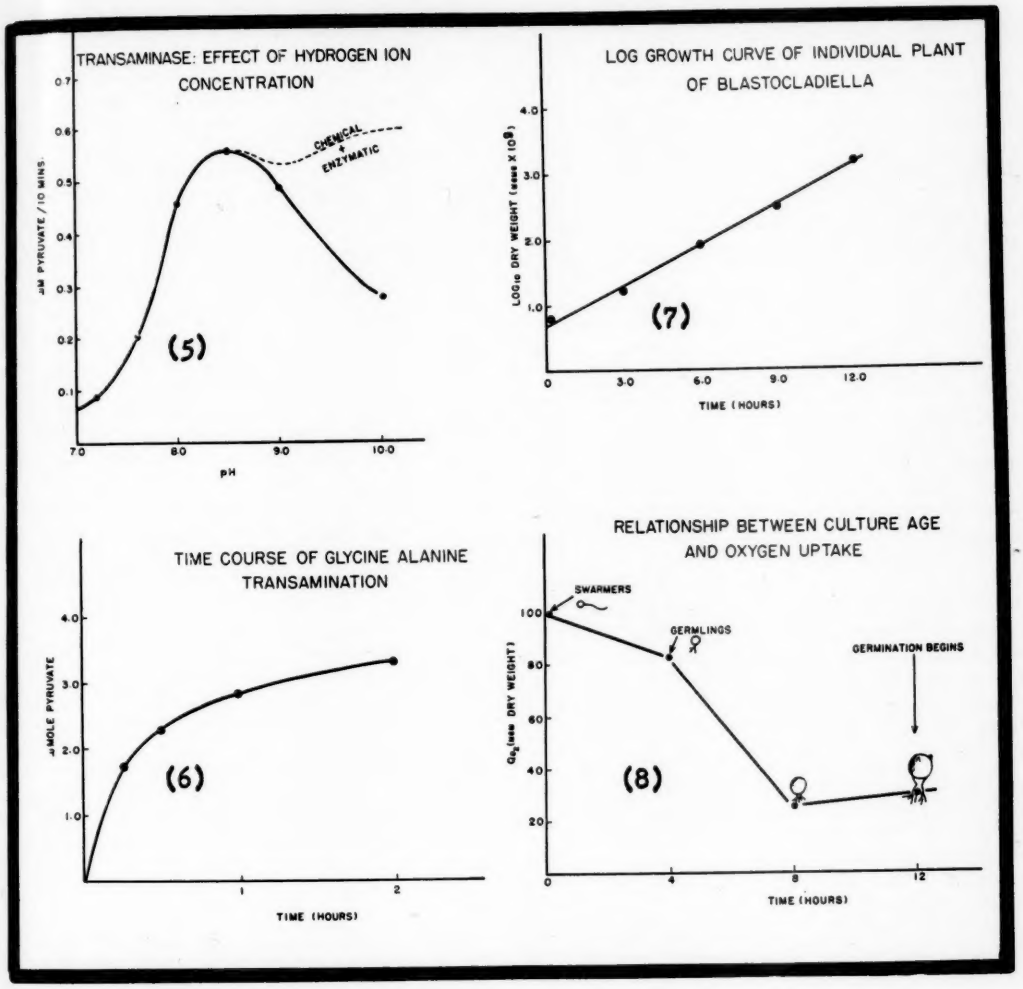



FIG. 5. Transaminase pH optimum. Conditions as for transaminase assay (table III) except 40 μM tris buffer used from pH 7.2 to 8.4, and 40 μM borate buffer from pH 8.4 to 10.0. In addition to the usual boiled enzyme controls, chemical controls were incubated for each pH, and pyruvate determined using the pH 7.2 reaction mixture as control. Enzyme: Gel supernatant B.

FIG. 6. Time course of glycine-alanine transamination. Conditions as for transaminase assay (table III) except that incubation times were as indicated; reaction stopped with 0.09 ml 2 *N* HCl. Enzyme: Gel Supernatant B.

FIG. 7. Log-growth curve of individual plant of Blastocladiella. The plotted data are the logs of the values obtained by dividing the dry weights obtained at times indicated, by the corrected number viable swarmers in the inoculum.

FIG. 8. Relationship between culture age and oxygen uptake. $Qo_2$, μl $O_2$/mg dry wt/hr

TABLE V

INHIBITION OF TRANSAMINASE*

| ADDITIONS | μM PYRUVATE | % INHIBITION |
|---|---|---|
| Control | 0.65 | 0 |
| $10^{-3}$ M KCN | 0.63 | 0 |
| $10^{-2}$ M KCN | 0.05 | 92 |
| $10^{-3}$ M hydroxylamine | 0.00 | 100 |
| $10^{-4}$ M hydroxylamine | 0.38 | 42 |
| $10^{-4}$ M hydroxylamine and $10^{-3}$ M pyridoxal phosphate | 0.65 | 0 |

* Reaction mixture as in Table III, but with additions as indicated; enzyme: Gel supernatant B.

out with a number of substrate pairs, including the keto acids pyruvate, glyoxylate, oxaloacetate, alpha-ketobutyrate, and alpha-ketoglutarate, and the amino acids alanine, glycine, isoleucine, serine, valine, leucine, lysine, phenylalanine, proline, glutamine, asparagine, cysteine, arginine, tryptophane, threonine, aspartate, and glutamate. Products were detected by paper chromatography. L-Alanine could not be replaced by any other amino acid in systems containing glyoxylate; D-alanine was inactive. An enzymatic transamination between pyruvate and asparagine, however, was detectable, but further work is necessary to determine if this is due to the same or a different enzyme; structural considerations, however, suggest that a separate enzyme is involved. Finally, assays of transaminase activity were carried out under the conditions used previously for light and dark assays of isocitritase. No stimulation by light occurred.

ENZYMOLOGY AND ONTOGENY IN BLASTOCLADIELLA: *Cultivation of synchronous single generation cultures of OC plants.* Using heavy spore suspensions and continuous aeration, up to 4 g/1.5 l PYG of single-generation populations were obtained in 14 hours. It seemed likely that a time-course analysis of such cultures might reveal further clues to the function of isocitritase and glycine-alanine transaminase in ontogeny. In preparation for these studies, a growth curve was established for single generation cultures of OC plants, their protein content at various ages determined (table VI), and their overall morphology recorded photographically (fig 9).

TABLE VI

SOLUBLE PROTEINS DURING ONTOGENY

| AGE, HRS | SOLUBLE PROTEIN (MG/GM)* |
|---|---|
| 0 (Zoospores) | 371 |
| 3 | 393 |
| 6 | 350 |
| 9 | 380 |
| 12 | 312 |

* Protein determinations on 22,000 × G supernatants obtained from Omnimixer homogenates

Growth was exponential, as indicated by a log plot of the dry weight data (fig 7). Microscop examination revealed that at all stages, plants were very uniform in morphology and size (fig 9), except toward the very end of the generation time (ca 1[illegible] hours) when some plants began to germinate. was clear that such cultures resulted from very nea synchronous growth of single generations of OC plants.

ENDOGENOUS RESPIRATION OF ZOOSPORES AND DEVELOPING PLANTS. Most of the enzymatic activities associated with the Krebs cycle had been found in mature, OC plants (17). Because of this and the close association of isocitritase and $CO_2$ fixation wit the tricarboxylic acid cycle, we looked for some in cation of the type of metabolism exhibited by spores. The endogenous $Qo_2$ of spores (at 3 and pH 6.5–7.0) was 102. Addition of numer substrates did not affect the rate. Arsenite $10^{-4}$ M) inhibited $O_2$ uptake 45 %. At pH 5.5 onate inhibited completely at $2 \times 10^{-3}$ M, and 50 at $4 \times 10^{-4}$ M. Succinate, at 10 times the malona concentration, returned the $Qo_2$ to endogenous leve (table VII). Thus presumptive evidence was o tained for a functioning tricarboxylic acid cycle i zoospores. However, since addition of various substrates failed to stimulate the endogenous $Qo_2$, further investigations will be necessary.

Since Brown and Cantino (4) had found a much lower $Qo_2$ (about 9.0) for mature OC plants than re ported here for spores, $Qo_2$ determinations were don at various developmental stages of a single generation culture. The $Qo_2$ decreased greatly as the plants developed to maturity (fig 8). However, as was to be expected, it was somewhat higher than the values reported for multiple generation cultures which had contained appreciable numbers of dead plants.

TABLE VII

EFFECT OF INHIBITORS ON ZOOSPORE RESPIRATION*

| ADDITIONS | CONCENTRATION | $O_2$ UPTAKE (μL/HR) | % INHIBITION |
|---|---|---|---|
| None | ... | 56 | ... |
| Arsenite | $4 \times 10^{-4}$ | 31 | 44 |
| None | ... | 46 | ... |
| Malonate | $4 \times 10^{-4}$ M | 24 | 48 |
| Malonate<br>Succinate | $4 \times 10^{-4}$<br>$4 \times 10^{-3}$ M | 37 | 20 |
| Malonate<br>Succinate | $4 \times 10^{-4}$ M<br>$8 \times 10^{-3}$ M | 46 | None |

* Flask contained 100 μM glycyl glycine, pH 5.5, 0.4 ml swarmer suspension, 0.2 ml 10 % KOH in center well, and additions as indicated, at 30° C. Glycyl glycine itself did not induce oxygen consumption.

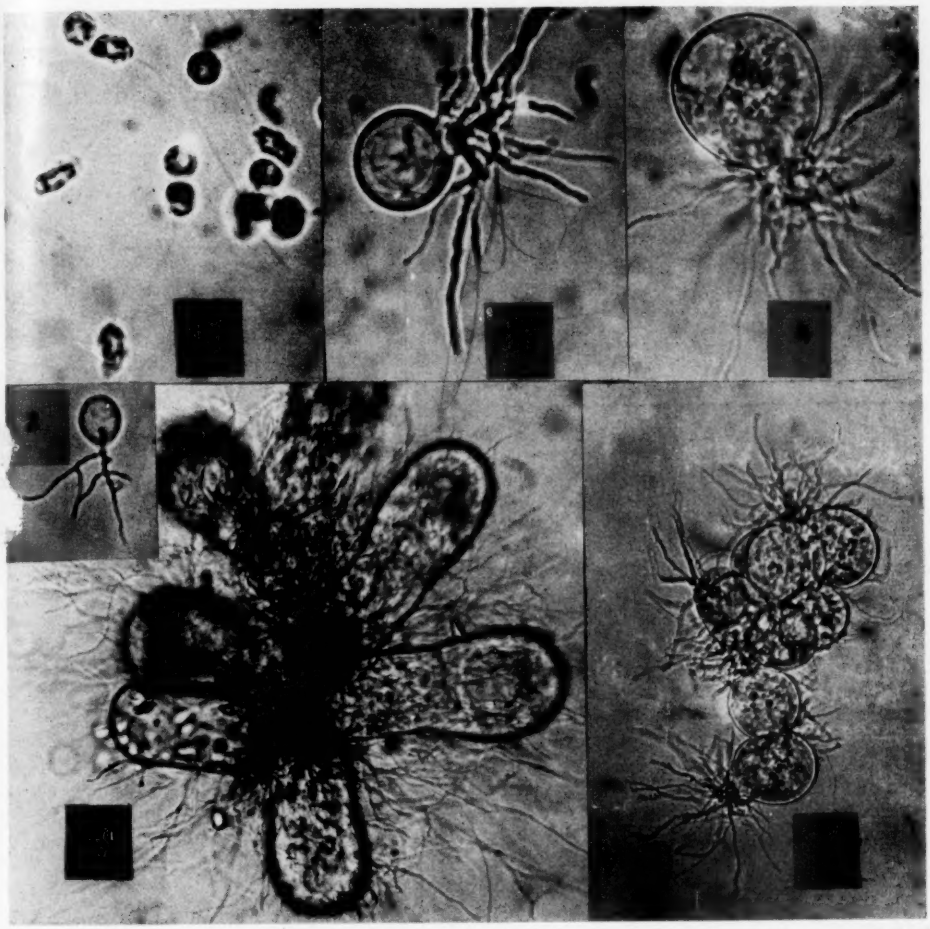


FIG. 9. (1), Zoospores from OC plants; note posterior flagella. (× 500). (2) to (6), plants derived from single-generation synchronous cultures. (2), 3-hour germling (× 500); (3), 6-hour OC plant (× 500); (4), 9-hour OC plant (× 500); (5), several 9-hour OC plants illustrating degree of uniformity of synchronous cultures (× 240); (6), 12-hour OC plants; note that one plant has almost completed release of zoospores (× 240).

RELATIONSHIP OF ISOCITRITASE AND TRANSAMINASE TO DEVELOPMENT AND MORPHOGENESIS. Isocitritase and transaminase activities were established for RS plants, OC plants, and spores. Because of high levels of endogenous amino acids, particularly alanine (38), it was not possible to assay zoospores directly for isocitritase; in this case, assays were made on spore extracts previously fractionated with 0.65 volumes of saturated $(NH_4)_2SO_4$. Both enzymes were detectable at all stages of the life cycle (table VIII).

Enzyme synthesis during growth of single generation cultures of OC plants was then traced in both light and dark. Enzyme activities per plant were calculated from conversion factors obtained from the growth curve data. Isocitritase (units/g dry wt and units/mg protein) decreased during the first 3 hours of growth, remained low until 9 hours, and then rose again to initial levels. But, the number of units *per plant* remained relatively constant and then increased almost exponentially during the last half of the generation time, thus demonstrating that *net synthesis of isocitritase does not occur until relatively late in development.* Although glycine-alanine transaminase drops somewhat (in units/g dry wt, and units/mg protein), it is apparently synthesized exponentially *from the very beginning of plant development!* The relationship between enzyme synthesis and over-all genesis of plant material is shown in figure 10.

TRANSAMINASE AND ISOCITRITASE IN DEVELOPING

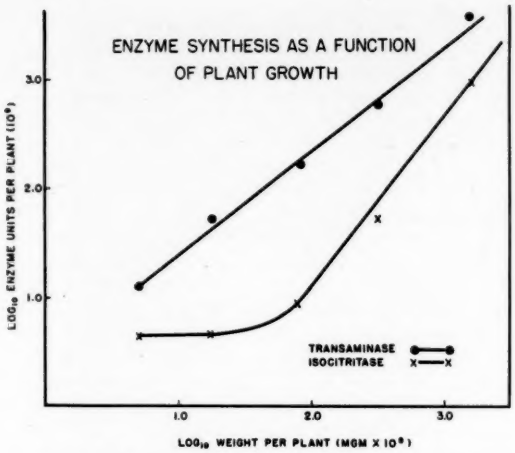
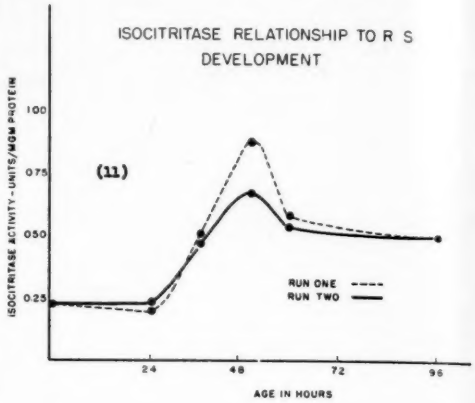



FIG. 10. Enzyme synthesis as a function of OC plant growth in the dark (data for light-grown plants were not significantly different). The plotted values were obtained from the log-data used in figure 7, and the specific activities of isocitritase and transaminase at different stages of development.

RS PLANTS. Finally, synchronous cultures of RS plants (38) were assayed as before for isocitritase and transaminase activities. The specific activity of isocitritase remained fairly constant during the first 24 hours (fig 11). However, beginning at 36 hours, there was a sharp increase in activity which peaked at 48 hours and then slowly declined. The specific activity at 48 hours was higher than that at any other stage in the life cycle (see table VIII). Transaminase activity behaved similarly (fig 12). Since RS plants mature (38) under these conditions at about 84 hours, the levels of activity of both enzymes reach their maxima at about 3/5 of the generation time!

## DISCUSSION

The purpose of this work was the study of growth and differentiation in terms of the underlying biochemical mechanisms involved. Enzymological studies of aquatic fungi have been rare, and have never before involved the study of enzymes at the degree of purity achieved for the glycine-alanine transaminase and isocitritase of *B. emersonii*. Therefore, before considering their possible roles in light-stimulated growth and morphogenesis, a brief discussion of the properties of these enzymes is appropriate.



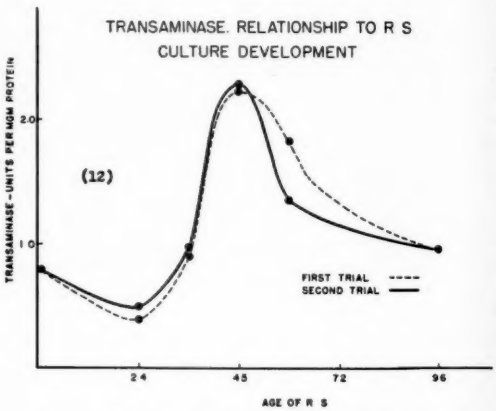




FIG. 11. Relationship between isocitritase activity and RS development.

FIG. 12. Relationship between transaminase activity and RS development, (age, in hrs).

TABLE VIII

DISTRIBUTION OF ISOCITRITASE AT VARIOUS STAGES OF LIFE CYCLE*

| STAGE OF DEVELOPMENT | ISOCITRITASE | | TRANSAMINASE | |
|---|---|---|---|---|
| | UNITS/G DRY WT | UNITS/MG PROTEIN | UNITS/G DRY WT | UNITS/MG PROTEIN |
| Zoospores | 82 | 0.23 | 271 | 0.79 |
| Mature OC plants | 55 | 0.18 | 193 | 0.58 |
| 4-day RS plants | ... | 0.50 | ... | 0.99 |

* Conditions as for assays of respective enzymes described previously.

ISOCITRITASE. Evidence for a deviation from the Krebs cycle involving formation of glyoxylate and succinate from tricarboxylic acids was provided by Campbell et al (8) using *Pseudomonas aeruginosa* (Schroeter) Migula, but they were unable to demonstrate the specific substrate for the reaction. However, the results of others (53, 50, 44) have since shown D-isocitrate to be the substrate for the reaction catalyzed by isocitritase; its activity has been identified in aerobic and facultative bacteria (54, 63), certain fungi (45, 35), and the castor bean (34). It has been partially purified from Pseudomonas (55), and yeast (45). Aerobic conditions (54, 36) were required for optimum enzyme production by bacteria grown in acetate and mineral salts media. Glucose repressed isocitritase formation in facultative bacteria, and decreased its activity in aerobic organisms; therefore, it appeared to be adaptively formed in these organisms.

The isocitritase of Blastocladiella closely resembles that from other organisms where similar levels of purification have been achieved. Thus, though it differs slightly in its pH optimum (pH 7.4 vs 8.2 for Pseudomonas) and $(NH_4)_2SO_4$ solubility, its Michaelis constant ($4.8 \times 10^{-4}$) is almost identical to that of the enzyme from *Pseudomonas aeruginosa* (55). The Michaelis constant of the yeast enzyme (45), determined under somewhat different conditions, is $1.2 \times 10^{-3}$ M. Like the reaction catalyzed by the enzyme from other sources, Blastocladiella's isocitritase mediates a reversible reaction specific for the D-isomer. The isocitritases from Blastocladiella and Pseudomonas exhibit similar saturation curves for both Mg and cysteine, with maximum activities at $10^{-3}$ M. Worthy of mention is the fact that the response curve for cysteine exhibits a maximum followed by decreasing activity with increasing cysteine concentration, a phenomenon probably attributable to a chemical reaction between cysteine and glyoxylate. Brunel-Chapelle (5) reported such a reaction at pH 9.0 and above. We have found considerable disappearance of glyoxylate even at physiological pH in the presence of sufficiently large excess of cysteine. Olson (45) reported a similar observation in studies on yeast isocitritase.

TRANSAMINASE. Transaminases catalyzing amino group transfer from all natural amino acids have been described (40), but relatively few have been purified. Only recently has a highly purified glutamate-aspartate enzyme been obtained (29, 30). Reports of transaminases catalyzing interconversion of glyoxylate and glycine have been rare and, except for transamination-deamination enzymes (discussed by Meister, 40), none has been purified or thoroughly studied. Cammarata and Cohen (6) and Awapara and Seale (3) reported an enzymatic transamination between glyoxylate and glutamate catalyzed by rat tissue preparations. Wilson et al (62) demonstrated similar activity in plant tissues. More recently, an extract of Pseudomonas was observed (7) to catalyze transamination of glyoxylate to glycine from glutamate, aspartate, alanine, asparagine, and glutamine. It was not established, however, whether one or several enzymes were involved, and no fractionation was attempted.

Though alanine participates in a number of transaminations (49, 3, 47, 48, 1), there are no reports other than that of Campbell (7) of a glyoxylate-alanine transaminase.

Extracts of *B. emersonii* mediated the rapid conversion of glyoxylate to glycine by amino group transfer from alanine. Since this might have constituted a pulling reaction in the S.K.I. cycle (14), and since there have been no previous reports of attempts to purify a glycine-alanine transaminase, further studies were carried out. The enzyme, purified 80-fold, was quite stable and resembled other transaminases in exhibiting maximum activity at alkaline pH. Attempts to demonstrate direct stimulation by pyridoxal phosphate were unsuccessful. However, it seems likely, in view of its almost-universal role in transamination, that pyridoxal phosphate is involved in the glyoxylate-alanine transamination as well; inhibition of the reaction by hydroxylamine and its reversal by pyridoxal phosphate indicates that this is so. We have concluded that the coenzyme is probably very tightly bound to the holoenzyme, though Meister (40) pointed out the theoretical possibility of enzymatic transaminations involving glyoxylate not requiring a cofactor.

It was expected that the equilibrium of the transamination would be far in the direction of glycine production (41). However, conversion to glycine and pyruvate never exceeded about one-third conversion, while the reverse reaction yielding alanine was only detectable under certain conditions; therefore, a true equilibrium was never attained. Clearly, pyruvate had an inhibiting effect upon the enzyme. Nevertheless, there is no reason, a priori, to believe that the equilibrium constant of the enzymatic reaction should be different than that for chemical transamination involving glyoxylate.

ENZYMES AND DEVELOPMENT IN BLASTOCLADIELLA. Our primary interest in isocitritase was based upon indirect evidence (14, 15, 16) that it was involved in a mechanism for $CO_2$ fixation as the pulling reaction which removed isocitrate from the site of reductive carboxylation of ketoglutarate. The S.K.I. (14) mechanism had been thought to operate as follows: in the presence of bicarbonate, light energy helped to push or pull the reductive carboxylation of ketoglutarate to isocitrate and thence, via an enzyme (presumed to be isocitritase), to succinate and a $C_2$ fragment (presumed to be glyoxylate). It was thought that glyoxylate and some of the succinate produced were then integrated into the biosynthetic machinery which yielded increased growth under conditions of illumination. In morphogenesis leading to RS plants, if a similar mechanism is assumed to operate, the high concentration of bicarbonate involved (ca $10^{-2}$ M) must upset a critical, internal

balance so that instead of increased growth, an entirely new course of development is pursued culminating in the production of resistant sporangia. That this is the case now seems all the more likely in view of the observation (12) that the bicarbonate trigger mechanism is also stimulated by light. Moreover, because of the high specific activity of the transaminase as compared to isocitritase and the probable equilibrium (40, 41) of the glyoxylate-glycine conversion towards glycine production, it now seems a reasonable assumption that transamination constitutes one of the important reactions responsible for the further metabolism of glyoxylate. Therefore, experiments were carried out to elucidate the roles of these enzymes in Blastocladiella. Because the specific locus of the light effect had not been established, any reaction presumed to be involved in light stimulated growth was suspect. Tests on the purified enzymes themselves, however, established unequivocally that neither was the specific locus for the light effect.

A method was then developed for cultivating massive OC cultures which, on the basis of their synchronous nuclear division (58), morphological uniformity and the logarithmic nature of their growth curves, were very nearly synchronous for most of the generation time. Meanwhile, conditions were also established for growing synchronous cultures of RS plants (38). In addition, a new technique was developed that permitted for the first time, physiological studies of the motile spores of aquatic fungi. Thus, while previous studies of developmental problems were limited to the use of heterogeneous liquid cultures containing several generations of plants, now it has become possible to study much more precisely, biochemical changes as they occur from the germination of the tiny zoospores to the development of the mature plant along either of the alternative, ontogenetic paths.

Armed with these new techniques, we followed the behavior of isocitritase and glycine-alanine transaminase during the development of a single generation of OC plants. On a protein basis, the specific activity of isocitritase was high in the spores, dropped sharply as development began, remained low for about half the generation time, and then rose again to initial levels. Transaminase activity, on the other hand, changed relatively little during plant growth; it dropped only one-third from starting levels in the spores to those in mature plants. But, for purposes of interpretation, a much different and more meaningful picture evolved by considering the synthesis of the two enzymes per individual plant. Thus, glycine-alanine transaminase was synthesized exponentially throughout the generation time as a linear function of plant growth, while isocitritase synthesis exhibited a definite lag extending to between the 3rd and 6th hours after germination of the spores.

The possible significance of the lag in isocitritase synthesis during ontogeny may best be appreciated on the basis of comparative biochemistry. It is axiomatic that the cell must have some way of controlling its metabolism, and one means whereby this is accomplished is by control of enzyme synthesis. A great deal of work has been done on adaptive enzyme formation in microorganisms as a possible mechanism for control of enzyme synthesis. One significant finding has been the remarkable correlation between the need for particular enzyme functions and the synthesis of the enzymes involved. For example, facultatively anaerobic bacteria (24) and yeast (52), when cultivated anaerobically, apparently synthesized Krebs cycle enzymes at a very low rate; but aerobically, rapid synthesis of the whole array of respiratory enzyme activities was brought about.

Thus, on the basis of past studies and the preceding discussion, we have evolved the following working hypothesis: lack of synthesis of isocitritase during the early stages in development of an OC plant reflects a situation in which the reaction it catalyzes is not required. During the early phases of the generation time, increased nucleic acid synthesis (58) and other reactions associated with light-stimulated growth take place at the expense of a readily available source of metabolites which, at least in part, may be alternatively supplied by isocitritase if it is present. After 3 to 6 hours of growth, this pool of precursors becomes depleted or insufficient, necessitating either initiation of, or a shift to, an alternative mechanism of supply. Since this period in ontogeny corresponds to the sharp decrease in endogenous respiratory activity (fig 8), it is possible that the deficiency is due to decreased production of substances associated with a functioning Krebs cycle. In any case, in response to the requirement for an alternative supply route, synthesis of isocitritase is begun thus bringing into operation the S.K.I. cycle, with a consequent increase in $CO_2$ fixation, which is now pulled by light. The glyoxylate produced is at least in part transaminated with alanine to yield glycine which, in turn, may be used to produce thymine (23) and purines (26) for nucleic acid synthesis.

It is probably premature to try to discuss in great detail, with the comparative biochemical literature as a frame of reference, the possible metabolic fates of glyoxylate, succinate, and glycine in relation to differentiation and growth. However, the finding (16) that glyoxylate and succinate together, direct products of the isocitritase cleavage, will replace the light effect in vivo, and our discovery of a direct metabolic route from glyoxylate to glycine are strongly suggestive of one particularly likely route of metabolism. Thus, Kikuchi et al (33) reported an enzymatic reaction yielding delta-aminolevulinic acid from succinate and glycine which was stimulated by light. Moreover, studies with radioactive tracers have incriminated delta-aminolevulinic acid as an intermediate in the conversion of glycine to thymine and purines (51), as well as carotene synthesis (39); and this brings us to the observation (58) that thymine (but not uracil) will substitute for light in DNA synthesis in Blastocladiella. From these findings, we cannot help but regard as very attractive, the possibility that in Blastocladiella, delta-aminolevulinic acid

is an important link connecting glyoxylate, glycine, and succinate with light stimulated growth and, in RS morphogenesis, at least carotene synthesis.

Finally, in a preliminary attempt to elucidate the roles of isocitritase and transaminase in the alternate developmental pathway, synchronous cultures of RS plants were also studied. The results showed an extraordinary correlation between the specific activities of the two enzymes and the morphological and biochemical changes which occur during the genesis of a resistant sporangium. Beginning at about the 24th hour of ontogeny, the specific activities of both glycine-alanine transaminase and isocitritase increased sharply, reaching maximum levels at 48 hours; these levels were 3 to 4 times higher than at any other stage of the entire life cycle. Simultaneously, there is a dramatic appearance of gamma carotene and melanin, and increased synthesis of lipid and chitin, as well as other structural and physiological changes (38). All of these events occur at approximately three-fifths of the generation time, the very point of no return in Blastocladiella (10) beyond which differentiation is irreversible!

These results lend significant support to our contention that isocitritase and glycine-alanine transaminase are key components of both the light-stimulated growth of OC plants and the bicarbonate trigger mechanism in morphogenesis of RS plants.

## Summary

The enzyme, isocitritase, which had been postulated to play an important role in the development of *Blastocladiella emersonii*, has been purified about 50-fold from extracts of this organism and some of its properties have been studied. A second enzyme, glycine-alanine transaminase, which has not been previously described, was purified about 80-fold and its properties similarly examined. New methods were developed which permitted, for the first time among aquatic fungi, precise, enzymological studies at all stages of the synchronized growth of populations of Blastocladiella, from zoospores to the mature, ordinary colorless plants. The synthesis of isocitritase and glycine-alanine transaminase was followed during the development of both colorless and resistant-sporangial plants, and an attempt was made to integrate the results into an interpretation of the function of these enzymes in morphogenesis and lumisynthesis (light-stimulated growth) in *B. emersonii*.

## Literature Cited

1. Alternbern, R. A. and R. D. Housewright. 1953. Transaminases in smooth *Brucella abortus*, strain 19. Jour. Biol. Chem., 204: 159–167.
2. Arrhenius, S. 1915. Quantitative laws in biological chemistry. G. Bell and Sons, Ltd., London.
3. Awapara, J. and B. Seale. 1952. Distribution of transaminases in rat organs. Jour. Biol. Chem. 194: 497–502.
4. Brown, D. H. and E. C. Cantino. 1955. The oxidation of malate by *Blastocladiella emersonii*. Amer. Jour. Bot. 42: 337–341.
5. Brunel-Chapelle, Genevieve. 1952. Sur le metabolisme de l'acid glyoxylique. Comptes rend. acad. sci., France 234: 1466.
6. Cammarata, P. S. and P. P. Cohen. 1950. The scope of the transamination reaction in animal tissue. Jour. Biol. Chem. 187: 439–452.
7. Campbell, L. L. 1956. Transamination of amino acids with glyoxylic acid in bacterial extracts. Jour. Bacteriol. 71: 81–83.
8. Campbell, J. J. R., R. A. Smith, and B. A. Eagles. 1953. A deviation from the conventional tricarboxylic acid cycle in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 11: 594.
9. Cantino, E. C. 1951. Metabolism and morphogenesis in a new Blastocladiella. Antonie v. Leeuwenhoek. 17: 325–362.
10. Cantino, E. C. 1952. The biochemical nature of morphogenetic patterns in Blastocladiella. Amer. Naturalist 86: 399–404.
11. Cantino, E. C. 1956. The relation between cellular metabolism and morphogenesis in Blastocladiella. Mycologia 48: 225–240.
12. Cantino, E. C. 1957. Light stimulated growth and $CO_2$ fixation in a chlorophyll-less fungus, Blastocladiella. Proc. of 2nd. International Congress of Photobiology, Torino. Pp. 453–456.
13. Cantino, E. C. 1960. Relations of metabolism to cell development. In: Handb. d. Pflanzenphysiologie, XV, W. Ruhland, ed. Springer-Verlag, Heidelberg, Germany. (in press).
14. Cantino, E. C. and E. A. Horenstein. 1956. The stimulatory effect of light upon growth and $CO_2$ fixation in Blastocladiella. I. The S.K.I. cycle. Mycologia. 48: 777–799.
15. Cantino, E. C. and E. A. Horenstein. 1957. The stimulatory effect of light upon growth and $CO_2$ fixation in Blastocladiella. II. Mechanism at an organismal level of integration. Mycologia. 49: 892–894.
16. Cantino, E. C. and E. A. Horenstein. 1959. The stimulatory effect of light upon growth and $CO_2$ fixation in Blastocladiella. III. Further studies, in vivo and in vitro. Physiol. Plantarum 12: 251–263.
17. Cantino, E. C. and M. T. Hyatt. 1953. Further evidence for the role of the tricarboxylic acid cycle in morphogenesis in *Blastocladiella emersonii*. Jour. Bacteriol. 66: 712–720.
18. Cantino, E. C. and G. T. Turian. 1959. Physiology and development of lower fungi (Phycomycetes). Ann. Rev. Microbiol. 13: 97–124.
19. Cavallini, D. 1950. Analizi spetrrofotometrica dei 2,4-dinitrofenilidrazoni dei alcuni chetoacidi di importanza biologica. Ricerca Sci., 20: 803–807.
20. Colowick, S. P. and N. O. Kaplan. 1955. Methods in Enzymology, I. Academic Press, Inc., New York.
21. Dagley, S. and E. A. Dawes. 1955. Citridesmolase: its properties and mode of action. Biochim. Biophys. Acta. 17: 177–184.
22. Davison, A. N. 1956. The mechanism of the inhibition of decarboxylase by isonicotinyl hydrazide. Biochim. Biophys. Acta. 19: 131–140.
23. Elwyn, D. and D. B. Sprinson. 1954. The synthesis of thymine and purines from serine and glycine in the rat. Jour. Biol. Chem. 207: 467–476.
24. Englesberg, E. and J. B. Levy. 1955. Induced synthesis of tricarboxylic acid cycle enzymes as

correlated with the oxidation of acetate and glucose by *Pasteurella pestis*. Jour. Bacteriol. 69: 418–431.

25. Friedemann, T. E. and Gladys E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. Jour. Biol. Chem. 147: 415–442.
26. Goldthwait, D. A., R. A. Peabody, and G. R. Greenberg. 1956. On the occurrence of glycinamide ribotide and its formyl derivative. Jour. Biol. Chem. 221: 555–567.
27. Green, D. E., L. F. Leloir, and V. Nocito. 1945. Transaminase. Jour. Biol. Chem. 161: 559–582.
28. Greenwood, F. C. and A. L. Greenbaum. 1953. The spectrophotometric measurement of enzymatically-produced oxalacetic acid. Biochim. Biophys. Acta. 10: 623–626.
29. Jenkins, W. T. and I. W. Sizer. 1957. Glutamic-aspartic transaminase. Jour. Amer. Chem. Soc. 79: 2655–2656.
30. Jenkins, T. W., D. A. Yphanti, and I. W. Sizer. 1959. Glutamic-aspartic transaminase. I. Assay, purification and general properties. Jour. Biol. Chem. 234: 51–57.
31. Kay, R. E., D. C. Harris, and C. Entenmann. 1956. Quantification of the ninhydrin color reaction as applied to paper chromatography. Arch. Biochem. Biophys. 63: 14–25.
32. Keilin, D. and E. F. Hartree. 1938. On the mechanism of the decomposition of hydrogen peroxide by catalase. Proc. Roy. Soc. (London). B124: 397–405.
33. Kikuchi, G., D. Shemin, and J. Bachman. 1958. The enzymatic synthesis of d-aminolevulinic acid. Biochim. Biophys. Acta. 28: 219–220.
34. Kornberg, H. L. and H. Beevers. 1957. The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. Biochim. Biophys. Acta. 26: 531–537.
35. Kornberg, H. L. and J. F. Collins. 1958. The glyoxylate cycle in *Aspergillus niger*. Biochem. Jour. 68: 3p–4p.
36. Kornberg, H. L., A. M. Gotto, and Patricia Lund. 1958. Effect of growth substrates on isocitritase formation by *Pseudomonas ovalis* Chester. Nature. 182: 1430–1431.
37. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666.
38. Lovett, J. L. and E. C. Cantino. 1960. The relation between biochemical and morphological differentiation in *Blastocladiella emersonii*. II. Nitrogen metabolism in synchronous cultures. Amer. Jour. Bot. (in press).
39. Mackinney, G., C. O. Chichester, and T. Nakayama. 1955. The incorporation of glycine carbon into β-carotene in Phycomyces Blakesleeanus. Biochem. Jour. 60: xxxvii–xxxviii.
40. Meister, A. 1955. Transamination. Advances in Enzymology. 16: 185–246.
41. Metzler. D. E., Joanne Olivard, and E. S. Snell. 1954. Transamination of pyridoxamine and amino acids with glyoxylic acid. Jour. Amer. Chem. Soc. 76: 644–648.
42. Neilands, J. B. and P. K. Stumpf. 1958. Enzyme chemistry. Wiley and Sons, New York.
43. Ochoa, S. 1952. Enzymatic mechanisms of carbon dioxide fixation. In: The Enzymes, II, part 2, J. B. Sumner and K. Myrback, eds. Pp. 930–1032. Academic Press, New York.
44. Olson, J. A. 1954. The d-isocitric lyase system: the formation of glyoxylic and succinic acids from d-isocitric acids. Nature. 174: 695–696.
45. Olson, J. A. 1959. The purification and properties of yeast isocitric lyase. Jour. Biol. Chem. 234: 5–10.
46. Robertson, W. B. 1942. The preparation of sodium pyruvate. Science. 96: 93–94.
47. Rowsell, E. V. 1956. Transamination of pyruvate and other keto acids. Biochem. Jour. 64: 246–252.
48. Rudman, D. and A. Meister. 1953. Transamination in *Escherichia coli*. Jour. Biol. Chem. 200: 591–604.
49. Sallach, H. J. 1955. Evidence for a specific alanine-hydroxypyruvate transaminase. In: Amino Acid Metabolism, W. D. McElroy and B. Glass, eds. Pp. 782–787. Academic Press, New York.
50. Saz, H. J. and E. P. Hillary. 1956. The formation of glyoxylate and succinate from tricarboxylic acid by *Pseudomonas aeruginosa*. Biochem. Jour. 62: 563–569.
51. Shemin, D. 1956. The biosynthesis of porphyrins; the succinate-glycine cycle. In: Currents in Biochemical Research, D. E. Green, ed. Pp. 518–536. Interscience Publishers, New York.
52. Slonimski, L. 1953. La formation des enzymes respiratoires chez la levure. Editions Desoer, Liege.
53. Smith, R. A. and I. C. Gunsalus. 1954. Isocitritase: a new tricarboxylic acid cleavage system. Jour. Amer. Chem. Soc. 76: 5002–5003.
54. Smith, R. A. and I. C. Gunsalus. 1955. Distribution and formation of isocitritase. Nature 175: 774–775.
55. Smith, R. A. and I. C. Gunsalus. 1957. Isocitritase: enzyme properties and reaction equilibrium. Jour. Biol. Chem. 229: 305–319.
56. Smith, R. A., J. R. Stamer, and I. C. Gunsalus. 1956. Citritase and isocitritase: equilibria and energetics. Biochim. Biophys. Acta. 19: 567–568.
57. Stadtman, E. R., G. D. Novelli, and F. Lipmann. 1951. Coenzyme A function in and acetyl transfer by phosphotransacetylase system. Jour. Biol. Chem. 191: 365–376.
58. Turian, G. and E. C. Cantino. 1960. The stimulatory effect of light on nucleic acid synthesis in the mold *Blastocladiella emersonii*. Jour. Gen. Microbiol. 21: 721–735.
59. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques. Burgess Publ. Co., Minneapolis.
60. Waddell, W. J. 1956. A simple ultraviolet spectrophotometric method for the determination of protein. Jour. Lab. Clin. Med., 48: 311–314.
61. Wheat, R. W. and S. J. Ajl. 1955. Citritase, the citrate-splitting enzyme from *Escherichia coli*. I. Purification and properties. Jour. Biol. Chem. 217: 897–907.
62. Wilson, D. G., K. W. King, and R. H. Burris. 1954. Transamination reactions in plants. Jour. Biol. Chem. 208: 863–874.
63. Wong, D. T. O. and S. Ajl. 1955. Isocitritase in *Escherichia coli*. Nature 176: 970–971.
64. Youatt, J. 1958. The action of isoniazid on the transaminases of Mycobacterium tuberculosis (BCG). Biochem. Jour. 68: 193–197.

# RED DROP AND ROLE OF AUXILIARY PIGMENTS IN PHOTOSYNTHESIS

R. EMERSON AND E. RABINOWITCH[1]

PHOTOSYNTHESIS PROJECT, BOTANY DEPARTMENT, UNIVERSITY OF ILLINOIS

*Note: When Robert Emerson was killed in a plane accident on February 4, 1959, much of the experimental material accumulated in his two years work on the action spectrum of photosynthesis in the far-red region, remained unpublished. He was to present these results to the Botanical Congress in Montreal in August, 1959. Instead, the following paper was presented, prepared on the basis of Emerson's earlier talks and laboratory notes, and after consultation with his collaborators, R. V. Chalmers and C. Cederstrand. The theoretical discussion of the results is my own. The presentation of this paper was intended as a memorial to Dr. Emerson; I was urged to publish it to give all those working in the field access to the results of Emerson's last, exciting results. A paper presented by J. Myers (12) at the same meeting provides both confirmation and interesting expansion of the phenomenon which is becoming known as the Emerson effect*. In particular, it shows that the so-called chromatic transient, as observed after the replacement of illumination with light at 700 mμ by illumination with a shorter-wave light giving the same steady yield of photosynthesis, has the same action spectrum as the Emerson effect, i.e., suggests a specific role of chlorophyll b.*—E. RABINOWITCH.

## INTRODUCTION

From experiments on the action spectrum of photosynthesis (largely by Emerson and co-workers), and of chlorophyll fluorescence (by French, and Duysens) performed up to 1957, an apparently consistent picture emerged which could be summarized as follows.

Photosynthesis is sensitized, in algae of all divisions (and in the higher plants as well), most effectively by light quanta absorbed by chlorophyll a. Light absorbed by other, accessory pigments (chlorophyll b and c, the phycobilins, and the carotenoids) can contribute to photosynthesis with an efficiency which is either lower, or, at best, equal to that of the light absorbed by chlorophyll a. The efficiency of accessory pigments in sensitizing photosynthesis closely parallels their efficiency in sensitizing the fluorescence of chlorophyll a. It is therefore plausible to postulate that the contribution of accessory pigments to photosynthesis is based on transfer of the light energy absorbed by them to chlorophyll a (where it can be utilized to produce chlorophyll fluorescence, or to sensitize photosynthesis). A mechanism by which this transfer is likely to occur has been demonstrated by physicists (Perrin, Vavilov, Förster, and others), and is known as "resonance energy transfer" or "inductive resonance."

The efficiency of the energy transfer to chlorophyll from the several other pigments present in chloroplasts must depend mainly on two factors: the intimacy of their association, which may involve both spatial closeness and chemical attachment, and the overlapping of the fluorescence band of the accessory pigment and the absorption band of chlorophyll a (which is the measure of resonance between the two pigments).

If this picture is accepted, it can still be asked if the energy supply to chlorophyll a is the *main* purpose of the presence of the different accessory pigments in various photosynthetic organisms, or if some of them may have other physiological functions. In the case of the phycobilins, found mainly (but not only) in deep-water red algae, the energy-collecting function appears plausible and may be vital, because of the predominance, deep under the sea, of green light not effectively absorbed by chlorophyll. This fact, and the high efficiency of energy transfer from the phycobilins to chlorophyll (80–95 %), observed in measurements of the action spectrum of chlorophyll fluorescence, supports the assumption that energy supply is in fact the main raison d'etre of red (and blue) pigments in Rhodophyceae. The case of fucoxanthol appears similar; this pigment, too, contributes significantly to the absorption of submarine light by the Phaeophyceae and Bacillariophyceae, and experiments show a high efficiency of energy transfer from it to chlorophyll a. Other carotenoids, particularly those found in green plants, contribute very little to the total absorption of sunlight, and the efficiency of energy transfer from them to chlorophyll a is relatively low (20–50 %). It seems likely that the main physiological function of these pigments is different; the fact that they contribute excitation energy to chlorophyll a may well be merely a physically unavoidable, but physiologically insignificant phenomenon.

The above-mentioned picture had one dark spot. Blinks, Haxo, and Yocum (9) had concluded, from measurements of the action spectra of photosynthesis in various species of red algae, that the photosynthetic efficiency of the light quanta absorbed by the phycobilins is generally *higher*; often much higher, than that of the light quanta absorbed by chlorophyll a. This suggested direct sensitization of photosynthesis by the red pigment, without the intermediary of chlorophyll a—in contradiction to the above-described picture. However, Duysens (3) found that a similar phenomenon can be observed also in the excitation of the fluorescence of chlorophyll a in these algae; it, too, is excited *more efficiently* by light quanta absorbed by the phycobilins than by those absorbed by chlorophyll a itself. The hypothesis that the accessory pigments

[1] Received November 2, 1959.

* Perhaps, it should be called the "second Emerson Effect", the first being the carbon dioxide burst at the beginning of the illumination period.

contribute to photosynthesis by transferring their excitation energy to chlorophyll a thus seemed to be valid in this case, too; it was, however, paradoxical that light absorption by chlorophyll itself appeared to produce excited chlorophyll molecules less apt to sensitize photosynthesis, or to emit fluorescence, than the excited chlorophyll molecules obtained by energy transfer from the accessory pigments.

I. Red Drop. Emerson decided to investigate this paradoxical situation in 1958. The detailed investigation of the action spectrum of photosynthesis in Porphyridium, made in collaboration with M. Brody (1), changed the experimental picture substantially. The relative inefficiency of light absorbed by chlorophyll a in red algae proved to be limited to the part of the absorption spectrum above 650 mμ; while at 644 mμ (where the light absorption is divided about equally between chlorophyll and phycocyanin), the absorbed quanta were found to be generally more effective than the 546 mμ quanta, absorbed entirely by the phycobilins. In the extreme case (that of algae adapted to green light, which is absorbed predominantly by phycoerythrin), the two efficiencies were equal (cf. fig 1). This suggests a situation similar to that found in other divisions of algae, where the efficiency of the accessory pigments can equal, but not exceed, that of chlorophyll.

It remains to be seen whether a correction similar to that brought by M. Brody's measurements to the action spectrum of *photosynthesis* of red algae, must be applied also to the above-mentioned conclusions of Duysens et al, concerning the action spectrum of chlorophyll a *fluorescence* in red algae; in other words, whether or not the quantum yield of directly excited chlorophyll fluorescence also declines in these cells only above 650 mμ.

The puzzle of the action spectrum of photosynthesis in red algae was shifted by M. Brody's measurements

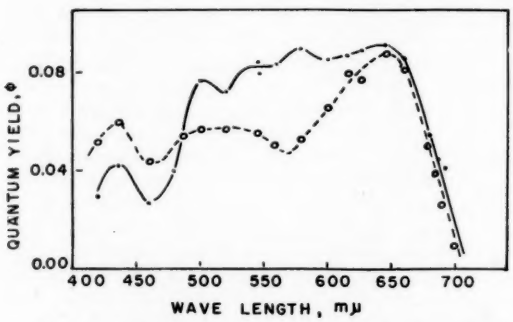



Fig. 1. Action spectra of photosynthesis in Porphyridium pre-illuminated with green light (solid line) and blue light (dashed line). In the first case, the phycobilins are about as efficient in sensitizing photosynthesis as chlorophyll; in the second case they are only about half as effective. There is no significant difference in the pigment content of the two samples. [After M. Brody and R. Emerson (1)].

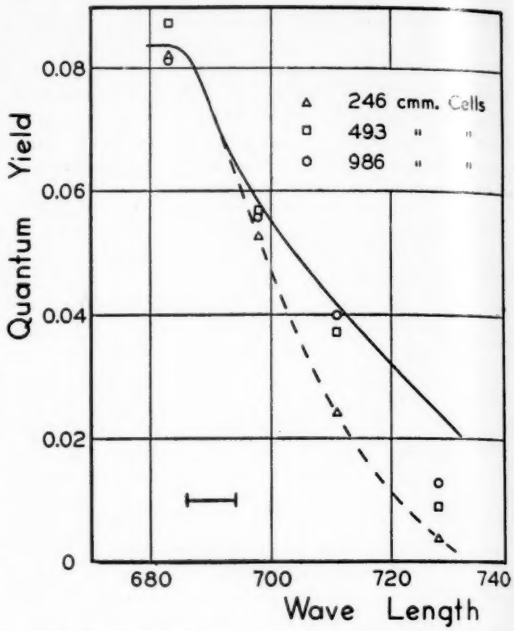



Fig. 2. Red end of the action spectrum of Chlorella. Solid curve is extrapolation of results to complete absorption. Width of spectral bands used is indicated. [After R. Emerson and C. Lewis (6)].

from the alleged low effectiveness of chlorophyll a *as such*, to the low efficiency of light absorbed by chlorophyll a $>$ 650 mμ. It seemed significant that this is the region where chlorophyll a becomes the *only* absorbing pigment; one had to recall the observation, made by Emerson and Lewis (6) in 1943, that in Chlorella, too, the quantum yield of photosynthesis declines in the extreme red part of the chlorophyll absorption band, $>$ 685 mμ—the region where the absorption by chlorophyll b is likely to be negligible, and practically all absorption must be due to chlorophyll a, (cf. fig 2). Emerson therefore conceived a working hypothesis, which seemingly ran contrary to the above-outlined generally accepted concept. He suggested that in order for photosynthesis to proceed with maximum efficiency, energy quanta must be contributed by *two pigments*: by chlorophyll a, *and* by one of the auxiliary pigments—chlorophyll b in green algae, a phycobilin in red algae, etc.

Although the red drop in the quantum yield of photosynthesis of green algae above 680 mμ was rather conclusively established by the 1943 study, the rapidly declining absorptive capacity of the cells in this region and their capacity for selective scattering of light on the long-wave side of the absorption peak (discovered by Latimer (10), cf. fig 3) permitted some doubt as to the reality of the drop; it could conceivably have been caused by overestimation of the number of quanta actually absorbed by chlorophyll in this region. A

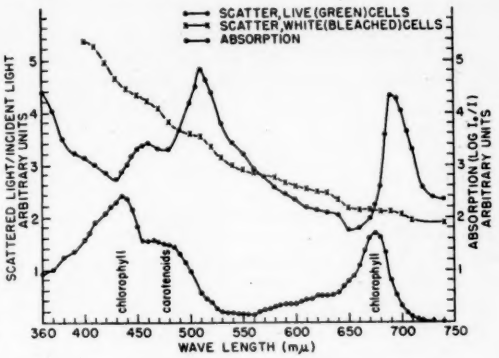
more precise study of the red drop was therefore undertaken by Emerson and Chalmers. Preliminary results have been presented in two communications to the National Academy of Sciences (5). Some speculations on the implications of the findings were discussed in a talk (4) before the Phycological Society. The main body of results remained unpublished at the time of Emerson's death.

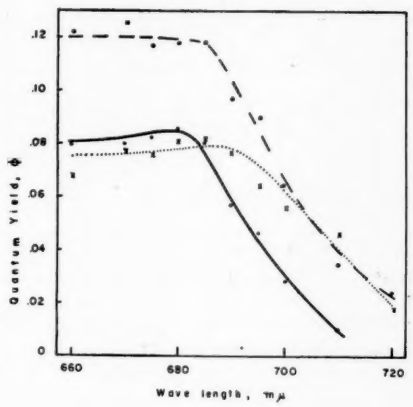
II. Enhancing Effect of Auxiliary Light. The far-red quantum drop made many people think that perhaps in this part of the spectrum, energy quanta may be too small to sustain photosynthesis, or at least to do so with a good yield. This seemed an attractive explanation, despite the fact that spectroscopic theory suggests that within a given electronic absorption band, the electronically excited molecules, after thermal equilibration with the medium (which is likely to be completed, in a condensed medium, before the photochemical reaction takes place), must have the same vibrational energy distribution, and thus also identical reactivity, whatever the specific size of the absorbed quantum.

If insufficient vibrational energy of the electronically excited chlorophyll molecules had something to do with the red drop, one could expect the quantum yield in this spectral region to improve with increasing temperature. The first experiments of Emerson et al (5) were therefore concerned with the effect of temperature on the quantum yield in the far red. A temperature effect was in fact found, but it had the opposite sign from that expected; the far red light was used more effectively at 5° C than at 20° C! (fig 4).

This finding, which in any case did not support the suggested interpretation of the red drop, was not further pursued, since another one, obtained at about the same time, appeared more exciting, supporting, as it did, the interpretation of the drop as indicative of the importance, for photosynthesis, of quanta absorbed by accessory pigments. This was the finding (figs 4, 5) that the quantum yield of photosynthesis in far red light can be substantially improved by auxiliary illumination with light of shorter wave length. The average quantum yield obtained in two superimposed beams proved to be substantially higher than the average of the quantum yields obtained by using the two light beams separately. Since in the auxiliary light per se, the yield was the usual one (8–12 quanta per molecule of $O_2$), while in the far red beam per se it was substandard (20–50 quanta per molecule of $O_2$), it was natural to ascribe the effect of the mixing of two lights to the improvement of the photochemical effectiveness of the quanta of far red light by the auxiliary light. (The average quantum requirement of two combined beams never was below the familiar minimum of 8 to 12 quanta.

The next question was: what was important, the *size* of the quanta of the auxiliary light, or the *pigment* that absorbed them? This required a determination of the action spectrum of the auxiliary light, a task that proved extremely laborious and time-consuming. The method used was to combine far red illumination



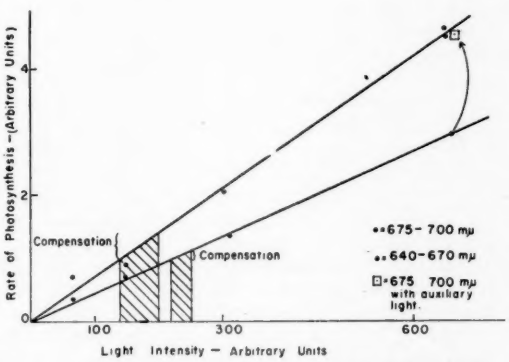




Fig. 3. Selective scattering of light by Chlorella cell suspension. [After P. Latimer and E. Rabinowitch (10)].

Fig. 4. Red drop of quantum yield of photosynthesis in Chlorella. Solid line, 5° C; dashed line 20° C; dotted line, 20° C with supplementary light of shorter wavelength. [After R. Emerson, R. V. Chalmers, and C. Cederstrand (5)].

Fig. 5. Proportionality of the rate of photosynthesis to light intensity in far-red and auxiliary light. [After R. Emerson, R. V. Chalmers, and C. Cederstrand (5)].

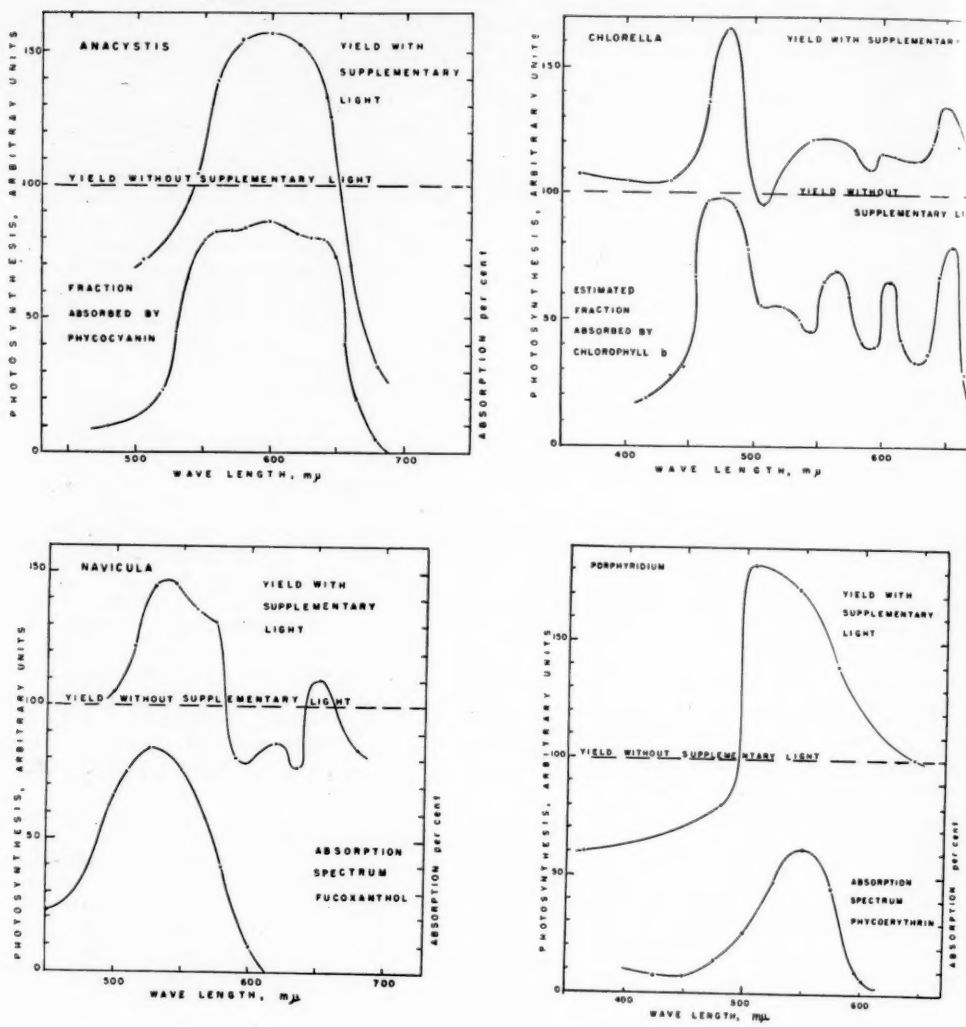



FIG. 6. Effect of monochromatic supplementary light on the yield of photosynthesis in far red light for the blue-green alga Anacystis, compared to the fraction of light absorbed by phycocyanin. [After R. Emerson, R. V. Chalmers, and C. Cederstrand, unpubl.].

FIG. 7. Effect of monochromatic supplementary light on the yield of photosynthesis in far-red light for the green alga Chlorella, compared to the fraction of light absorbed in chlorophyll b. [after R. Emerson, R. V. Chalmers, and C. Cederstrand, unpubl.].

FIG. 8. Effect of monochromatic supplementary light on the yield of photosynthesis in far-red light for the diatom Navicula, compared to the absorption spectrum of fucoxanthol. [after R. Emerson, R. V. Chalmers, and C. Cederstrand, unpubl.].

FIG. 9. Effect of monochromatic supplementary light on the yield of photosynthesis for the red alga Porphyridium, compared to the absorption spectrum of phycoerythrin. [after R. Emerson, R. V. Chalmers, and C. Cederstrand, unpubl.].

of certain constant intensity with the illumination by auxiliary light of an intensity giving, by itself, approximately the same amount of photosynthesis. The curve showing the yield of combined illumination, minus the yield caused by the auxiliary light alone, was plotted as function of wave length of the auxiliary light, and compared with the curve showing the relative proportion of light absorbed, at the same wavelengths, by the auxiliary pigment (or simply with the absolute absorption curve of this pigment).

Figures 6 to 9 show this comparison for algae of four divisions, containing phycocyanin, chlorophyll b, fucoxanthol, and phycoerythrin as main auxiliary pigment. In every case, the action spectrum of supplementary light is strikingly similar to the (relative or absolute) absorption spectrum of the auxiliary pigment.

The chosen method of comparison implied that the stimulating effect of auxiliary light on the far-red yield is proportional to (or at least, rises monotonously with) the intensity of the auxiliary light. This was checked by experiments with constant intensity of far-red, and varying intensity of auxiliary light. It clearly shows that the effect of auxiliary light is by no means a catalytic one [as suggested by Warburg (14) for the special case of blue light added to light of greater wave lengths], but rises with its intensity until it begins to show saturation, in the same intensity region where photosynthesis becomes light-saturated.

Considering the uncertainties involved in the calculation, the parallelism of the curve pairs in figures 6 to 9 appears striking. It justifies the conclusion that the enhancing effect of light of a certain wavelength on the yield in the far red parallels the proportion of light absorbed, at this wavelength, by the main auxiliary pigment—chlorophyll b in Chlorella, phycocyanin in Anacystis, fucoxanthol in Navicula, and phycoerythrin in Porphyridium. It seems that the far-red quanta, absorbed by chlorophyll a, are most effectively supplemented by quanta absorbed by an auxiliary pigment, and not by other quanta absorbed by chlorophyll a itself.

Since in an average higher plant a broad band of red and orange light, which is absorbed to about ¼ by chlorophyll b and about ¾ by chlorophyll a, can produce photosynthesis with full efficiency, it seems that quanta absorbed by chlorophyll a at the shorter wavelengths do not need supplementation by quanta absorbed by other pigments.

One matter that needs further attention is the appearance on figures 6 to 9, of *negative* effects. If confirmed by further experiments, they may mean that under certain conditions, combinations of far-red light with light of shorter wave lengths may decrease rather than increase the total yield (even though the experiments were carried out at light intensities far below saturation). In this case, it may be that the effect is due to decline in the efficiency of the auxiliary short-wave light to below the usual value of 8 to 10 quanta per $O_2$ molecule, rather than to a further decline in the efficiency of the far-red light. It is very likely that the need for balancing the excitation of the several pigments present in chloroplasts manifests itself in various ways, and not only in the inefficiency of far-red light absorbed only by chlorophyll a.

III. Experiments on Xanthophyceae (Heterokontae). According to data in the literature (13) Xanthophyceae contain no chlorophyll b. If Emerson's hypothesis is correct, they should show no effect of supplementary light on the yield in far-red light. This was in fact confirmed by experiments with Polyedriella. However, measurements of action spectra showed that this difference was due to the extension of high quantum yields out to 690 mμ, and *not* (as was expected) to the drop of the yield > 680 mμ being insensitive to auxiliary light! Since these algae were grown in a medium containing organic nutrients, it was suspected that the presence of such nutrients may permit full photosynthetic rate at 690 mμ, without the aid of supplementary light. Preliminary experiments by R. Govindjee suggested that if Polyedriella is precipitated from the organic and transferred into the inorganic medium, and the transfer is repeated several times, the red drop does appear after about five transfers. This suggests that Polyedriella grown in organic medium may contain a substrate (or a catalyst) which permits the cell to utilize light at 680 to 690 mμ with full efficiency, without the help of light of shorter wavelengths.

To test on Polyedriella the theory of chlorophyll b absorption as source of the auxiliary light effect on the yield in the far red, one would have to make the experiment on Polyedriella cultures repeatedly transferred into an inorganic medium; and this experiment has not yet been carried out.

IV. Effects of Organic Nutrition on Red Drop in Chlorella and Porphyridium. The observations on Polyedriella raised the question whether the red drop in other algae also could be affected by organic nutrition. Experiments with Chlorella grown in glucose solution showed no change, but algae grown in the presence of beef broth or earth extract did show a shift of the red drop to long wave lengths, (fig 10).

Because of Myers' findings in experiments on algal growth, it was suspected that vitamin K may be a possible active ingredient of broth or earth extract; experiments with the addition of this (and other) pure organic micronutrients are planned.

Even considerable alterations in the composition of the *inorganic* growth medium had no effect on the red drop in Chlorella.

V. Effect of Time Interval Between Absorption of Far-red Light and Supplementary Light. In the experiments described above, the experimental arrangement was that shown in figure 11. The far-red light, isolated by glass filters, entered the manometer vessel from above, while the auxiliary light came from below, usually through a monochromator (except in blue-violet light, where filters had to be

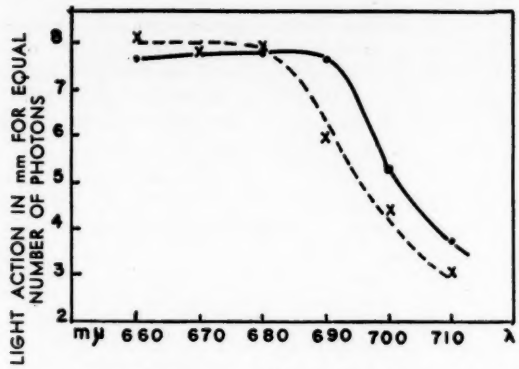



FIG. 10. Effect of earth extract on the red drop in Chlorella. [after R. Emerson and R. Govindjee, unpubl.].

used). In the majority of the experiments, both beams were practically completely absorbed. In a preliminary experiment, the auxiliary beam was sent from below and the far-red light from the side, and the enhancing effect was found to be considerably smaller. Since in the second arrangement, a cell needs less time to get from the bottom layer of the vessel, (where it has the greatest chance to absorb auxiliary light) into the layer where its chance to absorb far-red light is highest, this observation raised the possibility that a certain *time interval* between the two absorption acts may favor the effect. This suggests experiments in which the two beams would be alternated at variable time intervals.

VI. INTERPRETATION OF RED DROP. Emerson's conclusion that photosynthesis required (or, at least, is enhanced by) energy absorption in an auxiliary pigment, permits of several hypothetical interpretations. The simplest one is to postulate that two (or more) different primary photochemical processes are involved in photosynthesis, and are preferentially sensitized by the several pigments. For example, one could imagine that photosynthesis requires both *photoreduction* (of $CO_2$ or of a substrate derived from it, such as a carboxylic acid), and *photoxidation* (of water or a substrate derived from it). One could describe essentially the same concept by saying that each hydrogen atom (or electron) must be photoactivated *twice* on its path from the ultimate donor, water, to the ultimate acceptor, carbon dioxide, and that each of these two steps is preferentially sensitized by one or the other of the pigments. To use a concrete example, one could imagine a mechanism of the following type:

$$\text{I.}\quad \text{Chl a} + H_2O \xrightarrow{h\nu} O_2 + \text{r Chl a}$$

$$\text{II.}\quad \text{Chl b} + CO_2 \xrightarrow{h\nu} \{CH_2O\} + \text{o Chl b}$$

$$\text{III.}\quad \text{o Chl b} + \text{r Chl a} \rightarrow \text{Chl b} + \text{Chl a}$$

—chlorophyll a serving preferentially as photoxidant and chlorophyll b (or another auxiliary pigment) preferentially as photoreductant (or vice versa), and a dark reaction, III, between the reduced and the oxidized pigment restoring the original system. (I say "preferentially" because in the steady state, reactions I and II must proceed at the same rate: if only Chl b could sensitize reaction II, the maximum yield could be obtained only in light absorbed to one-half by chlorophyll b, and one-half by chlorophyll a, and not in a broad red-orange band absorbed to only one-fourth by the b component.)

The above interpretation of Emerson's findings is made unlikely, if not impossible, by the evidence, derived from fluorescence experiments, that of the quanta absorbed by chlorophyll b (as well as by phycobilins, or fucoxanthol), a very large fraction is transferred to chlorophyll a by resonance. This evidence is not as precise as could be desired, and the relevant experiments need to be repeated with improved techniques; but on the whole, they seem convincing.

It would be very difficult to explain the approximate equality of the quantum yields of fluorescence of chlorophyll a when excited by light absorbed by this pigment itself and when excited by light absorbed by chlorophyll b (or fucoxanthol) without assuming that most of the quanta absorbed by these auxiliary pigments are transferred to chlorophyll a. (True, the actual fluorescence yield is only, $< 3\,\%$, but it is a reliable index of what happens to the totality of the absorbed quanta).

An alternative interpretation of Emerson's results is to begin with the fact that most of the quanta absorbed by the auxiliary pigments are transferred to chlorophyll a, and thus cannot produce directly any specific photochemical effects. Instead, chlorophyll

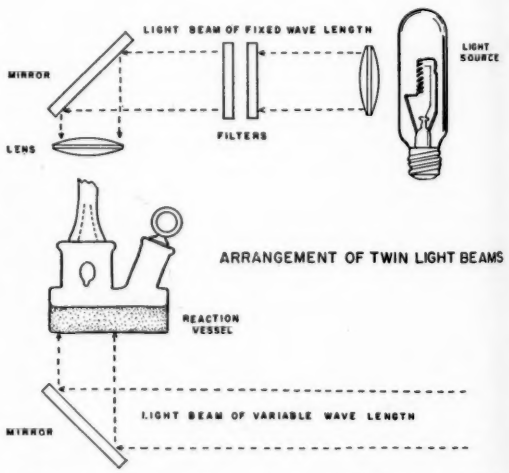
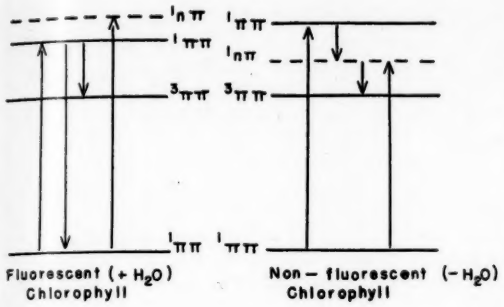



FIG. 11. Arrangement of two light beams in experiments with antiparallel beams. [after R. Emerson, unpubl.].



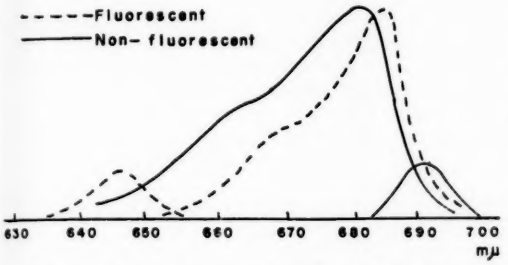




FIG. 12. Order of energy levels in fluorescent and non-fluorescent forms of chlorophyll. (Lowest $^1\pi\pi$ level, S, higher $^1\pi\pi$ level, S; $^3\pi\pi$ level is T.)

FIG. 13. Possible shapes of the absorption curves of chlorophyll in the fluorescent state (dashed line) and in the non-fluorescent state (solid line). In the first case, the n $\pi$ band lies on the short-wave side of the main $\pi\pi$ band; in the second case, it lies on its long-wave side, providing a possible explanation of the red drop.

a, carrying the transferred quanta, must be postulated to bring about preferentially a primary photochemical process different from the one caused (preferentially) by the long-wave quanta absorbed by chlorophyll a itself. To make this plausible, one could postulate, for example, the existence of two types of chlorophyll a complexes in the cell, e.g., such associated with a reductant and such associated with an oxidant, and assume that one of these two types is closer to the auxiliary pigment than the other.

Somewhat awkward is the necessity to postulate equal fluorescence yield of both types. Livingston (11) has shown how different is, for example, the fluorescence of chlorophyll associated with polar molecules (water, alcohol, amines) from that of chlorophyll surrounded exclusively by non-polar lipoid molecules. The fluorescence efficiency is generally determined by competition between the radiative transfer from the fluorescent (excited singlet) state to the ground singlet state, and the non-radiative transfer to the metastable triplet state (fig 12). It has been often postulated that the photochemical process is initiated by long-lived molecules in the triplet state; this explains why it often does not compete with fluorescence, i.e., why the substrate of the photochemical reaction often does not quench fluorescence. (This does not mean that photochemical processes *cannot* be initiated by the S*-state—in which case the substrate will quench the fluorescence).

The result of the competition between the processes S* → S and S → T in figure 12 can be strongly affected by the presence of a third, (so-called n $\pi$) excited state. If this state is located *between* the S*-and the T-state, its presence catalyzes the conversion to triplet, and thus reduces or altogether quenches fluorescence. If the n $\pi$-state is located *above* the S*-state, it has no such effect. This is the presently accepted explanation of the enhancing effect of polar molecules on the fluorescence of chlorophyll: the proximity of polar molecules shifts the n $\pi$-state upwards and the S*-state downwards, thus favoring fluorescence. In the hypothesis we are now considering, the two forms of chlorophyll complexes must both belong to the same, fluorescent type.

Franck (7) suggested a different interpretation. In it, the two kinds of chlorophyll involved in photosynthesis are a non-fluorescent form, in which the excited molecules in the S*-state are instantaneously converted, via the n $\pi$-state, into metastable molecules T; and a fluorescent kind, which permits the S*-state to survive long enough either to fluoresce, or to migrate, by resonance, through a sequence of chlorophyll molecules, until transferred to a molecule already in the T-state, raising it into an *excited triplet* state, T*. The latter has an energy content so high as to permit direct sensitization of an electron (or hydrogen) transfer from $H_2O$ to RCOOH (or their equivalents on the energy scale).

Absorption on the long-wave side of the main absorption peak, > 690 mμ, is suggested, in Franck's theory, to be due predominantly to n $\pi$ transitions in the non-fluorescent state (fig 13) it therefore produces predominantly T-molecules, which *alone* cannot bring about photosynthesis. Absorption in the main band, 650 to 690 mμ, on the other hand, excites both

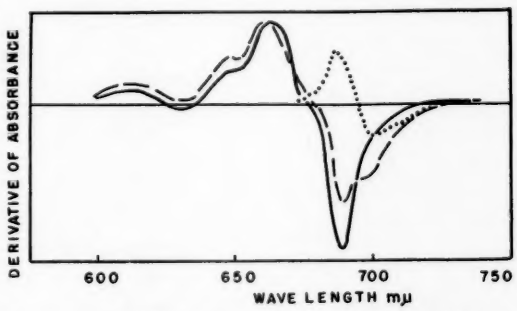



FIG. 14. Differential spectra of young and old cultures of Euglena suggesting the existence in vivo of a chlorophyll form with absorption peak at 695 mμ [after C. S. French (8)].

types of chlorophyll. Depending on their ratio, it can either produce about equal numbers of S* and T-molecules, and thus ultimately, a large number of T*-molecules and a high yield of photosynthesis (as in Chlorella), or predominantly T-molecules, and thus a low yield of chlorophyll fluorescence and photosynthesis (as in Porphyridium). Finally, (developing Franck's interpretation so as to fit M. Brody's findings), one can suggest that absorption at 640 to 650 mμ could excite predominantly the fluorescent species (in which the absorption on the short-wave side of the main peak is reinforced by the n $\pi$-transition!), and thus give, in all cells, a high yield of fluorescence and of photosynthesis.

It is to be noted that under no condition is there a danger of *shortage* of molecules in the T-state, since even in the extreme case of exclusive excitation of fluorescent molecules, the transition S* → T is the most likely fate of each S*-molecule, *unless* enough T-molecules have accumulated for the transition S* + T → S + T* to become equally likely.

Another note: as Franck pointed out, similar kinetic relationships can be derived also by considering, instead of a double excitation leading to the T*-state, two separate primary photochemical processes, one sensitized by molecules in the S*-state and one sensitized by molecules in the T-state, with the full quantum yield being only possible if the rates of the two processes are equal.

The assumption that there are (at least) two different kinds of chlorophyll in the chloroplasts imposes itself quite independently from Franck's ingenious hypothesis. The width of the red band in vivo alone suggests it (Duysens, personal communication). The analysis of the shape of the absorption band by French (8) provides quantitative evidence that the red band has a complex nature, and that its composition varies from species to species. One minor band, found in the absorption spectrum of some cells by French, has a peak at about 695 mμ, and could thus qualify as the band responsible for the drop in quantum yield of photosynthesis in Chlorella > 685 mμ (fig 14); it may be the n $\pi$ band of the non-fluorescent chlorophyll component postulated by Franck.

However, French also noted (in Euglena cells) a *fluorescence* band at 705 mμ, which he associated

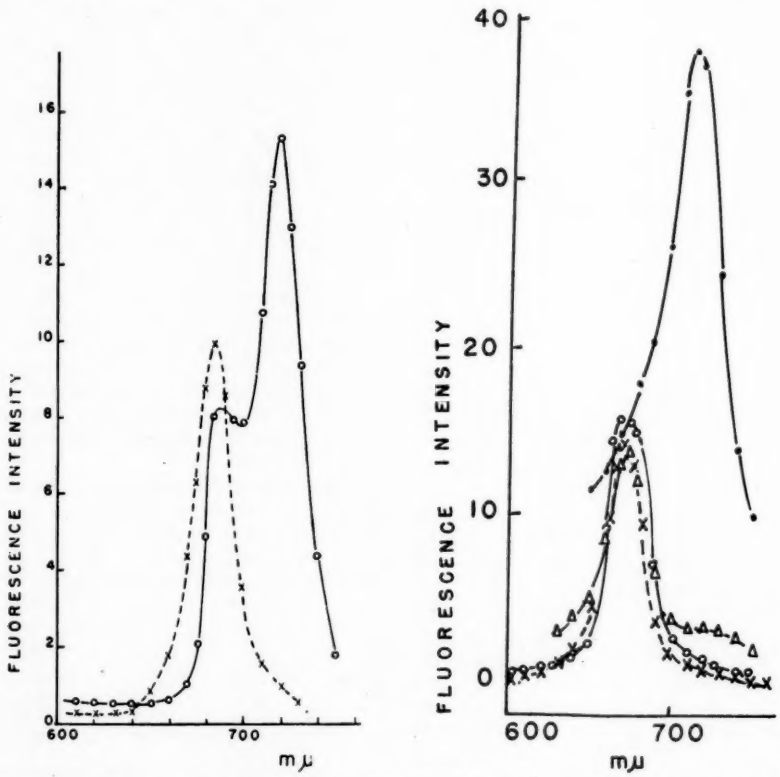



FIG. 15. Fluorescence spectrum of Chlorella at room temperature (dashed line) and at −193° C. (solid line). [after S. S. Brody (*2*)].

FIG. 16. Fluorescence spectra of chlorophyll a solutions in ethanol. Dots: concentrated solution at −193° C; triangles, same solution at room temperature; circles, dilute solution at −193° C; crosses, same solution at room temperature. [after S. S. Brody (*2*)].

with the 695 mμ absorption band. S. Brody (2) found a fluorescence band in the same position in low-temperature (liquid air) fluorescence spectrums of Chlorella (fig 15), and of concentrated (but not of diluted!) chlorophyll solutions (fig 16), and attributed it to dimeric (or higher polymeric) chlorophyll molecules. There is no doubt that chlorophyll, similarly to other dyes, does dimerize in concentrated solutions. From concentration quenching data, Weber (personal communication) calculated a dimerization constant of 4.5 for chlorophyll a in ether, which implies about 3 % dimerization at $10^{-2}$ m/l. The proportion of dimers may increase considerably at low temperature, and it is not implausible that dimeric molecules, non-fluorescent at room temperature, may become fluorescent at −190° C. To what extent dimerization can be expected to occur in chlorophyll in vivo, which is probably adsorbed on protein as a monomolecular layer, is uncertain. Brody suggested that the red drop in the action spectrum of photosynthesis is caused by dimers (or higher polymers) with an absorption band at 695 mμ; and that the much earlier drop in red algae could be explained by the same dimers being present in a much higher proportion.

Whatever specific hypothesis would be supported by future studies, it is highly plausible that the red drop is associated with the existence of several forms of chlorophyll a in vivo, and with the incapacity of one of these forms (or, to be more cautious, of one absorption band of this form) to sensitize photosynthesis with full maximum quantum yield; the enhancing effect of light absorbed by other pigments is most probably due to the preferential transfer of excitation energy from these pigments to another more active form of chlorophyll a.

## Summary

The enhancement of the quantum yield of photosynthesis in far red light (>680 mμ) by auxiliary light of shorter wavelengths is measured as a function of the wavelength of auxiliary light, for green (Chlorella), brown (Navicula), red (Porphyridium), and blue-green algae (Anacystis). The resulting "action spectra of the Emerson effect" show sharp peaks in the regions where the contribution of auxiliary pigments (chlorophyll b in Chlorella, chlorophyll c, and fucoxanthol in Navicula, phycoerythrin in Porphyridium, phycocyanin in Anacystis) to total light absorption is highest. This appears offhand as evidence that excitation of chlorophyll a alone is insufficient to produce photosynthesis (at least, not with a high yield), and simultaneous excitation of one of the auxiliary pigments is needed. However, the high yield of chlorophyll fluorescence in vivo sensitized by auxiliary pigments contradicts this interpretation, since it suggests highly efficient transfer of excitation energy from auxiliary pigments to chlorophyll a. A solution can be sought in the assumption of (at least) two different forms of chlorophyll a, which need to be excited to produce photosynthesis. Only one is excited by far red light, the other can be excited either directly, e.g., by near red light, or indirectly, by energy transfer from the auxiliary pigments.

## Literature Cited

1. Brody, M. 1958. Thesis, Univ. of Illinois. Brody, M. and R. Emerson. 1959. The quantum yield of photosynthesis in *Porphyridium cruentum*. Jour. Gen. Physiol. 43: 251.
2. Brody, S. S. 1958. A new excited state of chlorophyll. Science 128: 838–839.
3. Duysens, L. N. M. 1952. Transfer of Excitation Energy in Photosynthesis. Thesis, Univ. of Utrecht.
4. Emerson, R. and R. V. Chalmers. 1958. Speculations concerning the function and phylogenetic significance of the accessory pigments in algae. Phycol. Soc. Amer. News Bull. 11: 51–56.
5. Emerson, R., R. V. Chalmers, and C. Cederstrand. 1957. Some factors influencing the long-wave limit of photosynthesis. Proc. Nat. Acad. Sci., U.S. 43: 133–143. Abstract: Dependence of yield of photosynthesis in long-wave red on wavelength and intensity of supplementary light. Science 125: 746. Also, Yield of photosynthesis from simultaneous illumination with pairs of wavelengths. Science 127: ~~3305~~. 1059
6. Emerson, R. and C. Lewis. 1943. The dependence of the quantum yield of Chlorella photosynthesis on wave length of light. Amer. Jour. Bot. 30: 165–178.
7. Franck, J. 1958. Remarks on the long-wavelength limits of photosynthesis and chlorophyll fluorescence. Proc. Nat. Acad. Sci., U.S. 44: 941–948.
8. French, C. S. 1958. Various forms of chlorophyll a in plants. Brookhaven Symposia in Biology 11: 65–73.
9. Haxo, F. T. and L. R. Blinks. 1950. Photosynthetic action spectra of marine algae. Jour. Gen. Phys. 33: 389–422.
10. Latimer, P. and E. Rabinowitch. 1956. Selective scattering of light by pigment-containing plant cells. Jour. Chem. Phys. 24: 480. 1959. Selective scattering of light by pigments in vivo. Archiv. Biochem. Biophys. 84: 428–441.
11. Livingston, R., W. F. Watson, and J. McArdle. 1949. Activation of the fluorescence of chlorophyll solutions. Jour. Amer. Chem. Soc. 71: 1542–1550. Livingston, R. and S. Weil. 1952. Activation of the fluorescence of chlorophyll solutions. Nature 170: 750–752. Cf. also, Evstigneev, V. B., V. A. Gavrilova, and A. A. Krasnovsky. 1949. Doklady Acad. Sci., USSR 66: 1133, 70: 261.
12. Myers, J. and C. S. French. 1960. Relationship between time course, chromatic transient, and enhancement phenomena of photosynthesis. Plant Physiol. (in process) 35: 963–969
13. Strain, H. H. 1951. The Pigments of Algae. In: Manual of Phycology G. M. Smith, ed. Chronica Botanica Co., Waltham, Pp. 243–262.
14. Warburg, O., G. Krippahl, W. Schroeder, W. Buchholz, and E. Theel. 1954. Zeits. Naturforsch. 8b: 164.

# EFFECTS OF GIBBERELLIN, KINETIN, THIOUREA, AND PHOTOMORPHOGENIC RADIATION ON MITOTIC ACTIVITY IN DORMANT LETTUCE SEED[1]

ALAN H. HABER AND HELEN J. LUIPPOLD

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY[2], OAK RIDGE, TENNESSEE

Lettuce seed germination can be stimulated, probably by distinct mechanisms, by any of the three widely studied morphogenic agents: gibberellin, kinetin, or red light (11). It has been shown that cellular expansion is both necessary and sufficient and that cell division is neither necessary nor sufficient for the initiation of lettuce seed germination (8). Thus it is now possible to study the effects of germination-stimulating agents on mitotic activity in embryos uncomplicated by germination and growth from expansion. In this paper, mitotic activity as it is affected by gibberellic acid (GA), kinetin, and light, as well as by thiourea, which also stimulates germination of lettuce seed (28), is studied.

## MATERIALS AND METHODS

Seeds (achenes) of lettuce (*Lactuca sativa* L., var. New York) were sown on filter papers moistened with appropriate solutions at pH 5.7 as previously described (8). Unless otherwise indicated, the Petri dishes were placed in closed copper sterilization cans that were submerged in a constant-temperature water bath. The seeds were thus in continuous darkness. Seeds given light treatments were similarly sown in dishes set upon a Cold-Cel truck plate with refrigeration coils through which water was circulated from a 50 gal, constant-temperature water bath. The temperature within the Petri dishes was thus maintained at approximately 31.5° C under the light filters hereafter described. The dishes were enclosed in wooden frames with windows consisting of light filters through which light shone from above. The light source was a multiple water-cooled refluxing lamp unit (33). Seven 150-watt incandescent reflector flood bulbs shone down through 6 cm of water. The red-light filter system was a Corning No. 2403 polished filter and a Plexiglas filter containing a 2 cm thick layer of $CuSO_4 \cdot 5H_2O$ (50 g per liter) plus 0.5 % $H_2SO_4$. The far-red filter system was a Corning No. 7–69 molded filter and a similar Plexiglas filter containing a layer of water 2 cm thick. The red filter system transmits light from 625 to 680 mμ, with a peak near 650 mμ. The far-red filter system (which has a total of 8 cm of water, including the water in the light source) transmits radiation from 710 to 1,150 mμ, with a peak near 820 mμ. Transmission at 740 mμ is 58 % that of the peak transmission. Transmission spectra of the individual components can be found in the following sources: Filters Nos. 2403 and 7–69, color filters catalog available from Corning Glass Works, Optical Sales Department, Corning, N. Y.; copper sulfate solution from Withrow and Price (34), and water from Curcio and Petty (3). The seeds received 1 and 57 microwatts/mm² of radiant energy under the red and the far-red filter systems, respectively. These light treatments were given continuously from the beginning of imbibition.

The criterion of germination was visual detection of radicle protrusion. Radicles (1–2 mm) from nongerminated seeds were excised and prepared as described previously (8). The apical 0.5 mm was examined for mitotic activity. An entire dish of seeds was sampled at one time for germination percentages and mitotic activity in nongerminated seeds. Once sampled, the dish was removed from the experiment.

Respiratory rates were determined by placing 50 seeds in 1.2 ml of solution in the main compartment of Warburg flasks. We placed 0.2 ml of 20 % KOH and a fluted piece of filter paper in the center well to measure oxygen consumption by the direct method (32).

The term "germination" is used here in the physiologic sense of Toole et al (30): ". . . the start of germination depends upon coupling of respiration to growth." Accordingly, seeds that undergo mitotic activity without expansion of the embryo are not designated as germinated. When an embryo undergoes visible expansion (apart from water imbibition) the seeds would be designated as germinated, even if mitosis had not yet begun.

## RESULTS

EFFECTS OF GA, KINETIN, AND THIOUREA ON MITOSIS AT 37° C: To be meaningful, studies on germination and on mitosis in nongerminated seeds should be conducted under identical experimental conditions. Also, there should be no bias from sampling dissimilar populations of nongerminated seeds. We have found these conditions can be satisfied for studies with kinetin and thiourea by incubating the seeds at 37° C in darkness. Both kinetin and thiourea stimulate germination under these conditions (fig 1). The inability of GA to stimulate germination confirms an earlier observation that GA is ineffective in breaking thermodormancy of lettuce seed (11). After 1 day, however, none of the seeds had germinated in any

[1] Received November 5, 1959.

[2] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

TABLE I

EFFECTS OF GERMINATION-STIMULATING CHEMICALS ON MITOTIC ACTIVITY IN NONGERMINATED SEEDS AFTER 1 DAY AT 37° C IN DARKNESS

| No. of mitotic figs | No. of radicles | | | |
|---|---|---|---|---|
| | Control | GA* | Kinetin* | Thiourea* |
| 0 | 63 | 64 | 45 | 64 |
| 1 | 1 | ... | 9 | 1 |
| 2 | 1 | ... | 5 | ... |
| 3 | ... | ... | 1 | ... |
| 4 | ... | 1 | 1 | ... |
| 5 | ... | ... | 1 | ... |
| 6 | ... | ... | 1 | ... |
| 7 | ... | ... | 1 | ... |
| 8 | ... | ... | ... | ... |
| 9 | ... | ... | ... | ... |
| 10 | ... | ... | 1 | ... |
| >10 | ... | ... | ... | ... |
| Total No. radicles examined | 65 | 65 | 65 | 65 |
| Total No. mitotic figures observed | 3 | 4 | 54 | 1 |
| Radicles with one or more mitotic figures (%) | 3.1** | 1.5 | 30.8** | 1.5 |

* Concentrations given in figure 1.
** Difference significant at level of $P = 10^{-4}$.

of the treatments. Consequently, seeds could be sampled at this time from all treatments for examination of mitotic activity uncomplicated by growth from expansion. After 2 days, none of the seeds had germinated in water or GA, and the germination percentages in kinetin and thiourea were only 0.8 and 5%, respectively. Thus, after 2 days, these nongerminated seeds could be sampled from the different chemical treatments with very little sampling bias resulting from the germination. After incubation for either 1 or 2 days, 65 radicles were excised from the nongerminated seeds in each treatment for examination of mitotic activity (tables I, II). For each chemical treatment, the distribution of mitotic figures among the radicles deviated from randomness, with a very great discrepancy between the observed number of radicles having no mitotic figures and the number expected from the Poisson distribution having the same mean number of mitotic figures per radicle. These comparisons, which are given only for the water controls in table II, confirm previous results discussed elsewhere (8). For our present purpose, this particular aspect of the deviation from randomness permits the application of contingency tests of the significance of differences (26) in mitotic activity between treatments by comparing the numbers of radicles having one or more mitotic figures detected.

It is apparent that mitotic activity is greater in kinetin than in water in the nongerminated seeds after either 1 or 2 days at 37° C in darkness. The kinetin treatment resulted not only in increased mitosis but also in increased cell division inasmuch as no binucleate cells were observed.

GA had no effect on mitotic activity after either 1 or 2 days. After 2 days mitotic activity was inhibited by thiourea. Since thiourea inhibits mitotic activity in this experiment even though it stimulates germination, we conclude that thiourea is an inhibitor of mitosis in these seeds where mitosis is uncomplicated by growth by expansion. Before concluding definitely that kinetin is a true cell-division factor and that GA can stimulate germination without directly stimulating mitosis, it is necessary to rule out the alternative explanations considered in each of the following three subsections.

STUDIES OF ACTION OF KINETIN AT HIGH TEMPERATURE: Sufficiently high temperatures can inhibit germination (2) or mitosis in nongerminated seeds (8). Accordingly, the actions of kinetin on mitosis (tables I and II) and germination (fig 1) in seeds at superoptimal temperatures could conceivably result from a general protection against high-temperature effects; if so, then the kinetin effects would not necessarily result from more-direct actions upon either germination or mitosis. This possibility has been tested in two types of experiments. First, we gave seeds various high-temperature treatments designed to prevent subsequent germination under continuous white light at 26° C. The results of one ex-

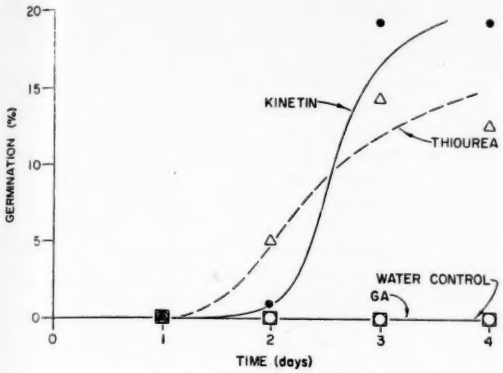
TABLE II

EFFECTS OF GERMINATION-STIMULATING CHEMICALS ON MITOTIC ACTIVITY IN NONGERMINATED SEEDS AFTER 2 DAYS AT 37° C IN DARKNESS

| No. of mitotic figures | No. of radicles | | | | Poisson distribution (refers to control) |
|---|---|---|---|---|---|
| | Control | GA* | Kinetin* | Thiourea* | |
| 0 | 38 | 38 | 9 | 61 | 0.5 |
| 1 | 4 | 3 | 3 | 2 | 2.4 |
| 2 | 2 | 2 | 4 | ... | 5.9 |
| 3 | ... | ... | 5 | 1 | 9.6 |
| 4 | 1 | 4 | 4 | .. | 11.7 |
| 5 | 4 | 1 | 4 | ... | 11.4 |
| 6 | ... | 1 | 1 | ... | 9.3 |
| 7 | 1 | ... | 2 | ... | 6.4 |
| 8 | 1 | 3 | 2 | 1 | 3.9 |
| 9 | ... | ... | 2 | ... | 2.1 |
| 10 | 2 | 2 | 3 | ... | 1.0 |
| 11 | ... | 1 | 2 | ... | 0.5 |
| 12 | 1 | 2 | 1 | ... | 0.2 |
| 13 | 1 | ... | 2 | ... | 0.1 |
| 14 | 1 | 1 | ... | ... | |
| 15 | ... | ... | ... | ... | |
| 16 | 1 | ... | 3 | ... | |
| 17 | 1 | ... | ... | ... | |
| 18 | 1 | ... | 1 | ... | |
| 19 | 1 | 1 | 1 | ... | |
| 20 | ... | ... | 1 | .. | |
| 21–22 | 1 | ... | 1 | ... | |
| 23–24 | 1 | 1 | 1 | ... | |
| 25–26 | ... | 1 | 2 | ... | |
| 27–28 | 1 | ... | 1 | ... | |
| 29–30 | 1 | 1 | 1 | ... | |
| 31–35 | ... | 1 | 1 | ... | |
| 36–40 | ... | 1 | 2 | ... | |
| 41–50 | 1 | ... | ... | ... | |
| 51–60 | ... | 1 | 2 | ... | |
| 61–70 | ... | ... | ... | ... | |
| 71–80 | ... | ... | 1 | ... | |
| 81–90 | ... | ... | 2 | ... | |
| 91–100 | ... | ... | 1 | ... | |
| >100 | ... | ... | ... | ... | |
| Total No. of radicles examined | 65 | 65 | 65 | 65 | 65 |
| Total No. of mitotic figures observed | 317 | 351 | 1,028 | 13 | 317 |
| Radicles with 1 or more mitotic figures (%) | 41.5[A] | 41.5[B] | 86.2[B] | 6.2[A] | |

* Concentrations given in figure 1.

[A, B] Pairs of figures with the same symbols significantly different from each other, $P = 10^{-4}$. The kinetin data are compared with the GA data rather than with the control data to avoid using the same observations for two different tests of significance. See table I for comparison between kinetin and control treatments.



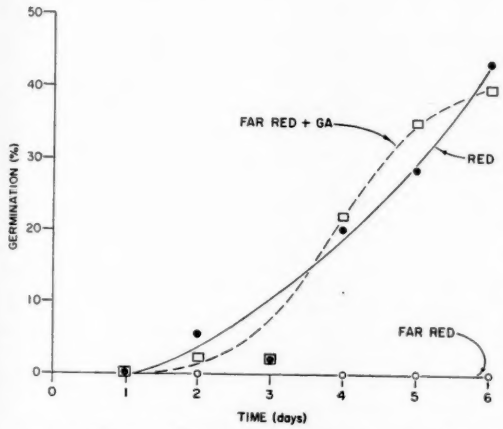




FIG. 1 (*Top*). Effects of GA, kinetin, and thiourea on germination at 37° C in darkness. ○, Water controls; □, $3 \times 10^{-4}$ M GA; ●, $10^{-5}$ M kinetin; and △, $3 \times 10^{-2}$ M thiourea. Each point represents the germination percentage of an independent sample of 120 seeds.

FIG. 2 (*Bottom*). Effects of GA and light on germination at 31.5° C. ○, Far-red; ●, red; and □, far-red + $3 \times 10^{-4}$ M GA. Each point represents the germination percentage of an independent sample of 88 to 94 seeds.

periment are given in table III. Seeds germinated directly gave 95 % germination, whereas seeds first kept 6 hours at 45° C gave only 21 % germination under the same conditions of continuous lighting at 26° C. Incubation in various concentrations of kinetin during the 6 hours at 45° C, however, did not significantly protect against the deleterious effect of the high-temperature treatment. These data suggest that kinetin does not generally increase tolerance of the seeds to high-temperature stress. Consequently, its effects on increasing mitotic activity in nongerminated seeds at 37° C should be regarded as a true acceleration of mitosis. Similarly, the effect of kinetin on germination at superoptimal temperatures should be regarded as a true breaking of thermodormancy rather than a protection of the germination mechanism against heat damage. A similar conclusion is also suggested by studies of the respiratory rates of seeds at 37° C in water or in kinetin. As shown in table IV, the respiratory rate per seed, which is a general indication of over-all metabolic activity, is unaffected by kinetin treatments which stimulate germination. Although all experiments were performed with the same batch of seeds, those cited in tables III and IV were done about 9 and 11 months, respectively, before the other experiments cited in this paper. The somewhat better germination rate of the kinetin-treated seeds (table IV compared with that shown in fig 1) may possibly be related to this and to other differences of experimental method necessitated by the manometric techniques.

SYNERGISTIC ACTION OF GA WITH EITHER KINETIN OR THIOUREA AT HIGH TEMPERATURE: The results given in tables I and II might suggest that GA generally has no effect on mitosis in seeds when such mitosis occurs without growth by expansion. Since treatment with GA alone also fails to allow any germination at this temperature (fig 1), the possibility remains that GA loses all its activity at the high temperature. Accordingly, the data in tables I and II in themselves do not permit the conclusion that GA has no direct effect on mitotic activity under those conditions for which it might still be active with respect to germination. The experiment summarized in table V confirms the ineffectiveness of GA alone to stimulate germination at high temperature. However, GA does retain the capacity to stimulate germination in the presence of either kinetin or thiourea.

TABLE III

EFFECT OF KINETIN ON HIGH-TEMPERATURE-INACTIVATION OF LETTUCE SEED GERMINATION

| HOURS AT 45° C* | KINETIN MOLARITY DURING HIGH-TEMP TREATMENT* | NO. SEEDS | SUBSEQUENT GERMINATION AT 26° C** |
|---|---|---|---|
| | | | (%) |
| 0 | ... | 240 | 95.4 |
| 6 | 0 | 579 | 21.2 |
| 6 | $3 \times 10^{-6}$ | 264 | 26.1 |
| 6 | $10^{-5}$ | 272 | 26.5 |
| 6 | $3 \times 10^{-5}$ | 134 | 13.4 |
| 6 | $10^{-4}$ | 285 | 21.4 |

* Batches of approximately 130 to 140 seeds were sown on one piece of filter paper moistened with 5 ml of appropriate solution in the bottom of 250 ml Erlenmeyer flasks. The opening of the flask was covered with Parafilm, and the main body of the flask was submerged in a 45° C water bath.

** After exactly 6 hours, seeds were washed out of each flask into a cheesecloth bag and repeatedly washed with distilled water. The seeds were then transferred to 9 cm Petri dishes containing one piece of filter paper moistened with 3.5 ml of water. Germination percentages were determined after 3 days at 26° C under continuous white light of 15 ft-c.

TABLE IV

EFFECT OF KINETIN ON RESPIRATION AND GERMINATION AT 37° C

| TIME* (HOURS) | $Q_{O_2}$ (MICROLITERS/50 SEEDS**) | |
|---|---|---|
| | WATER CONTROLS | 5 × $10^{-5}$ M KINETIN |
| 3–4 | 36.4 | 38.8 |
| | 39.7 | 37.5 |
| | 32.4 | 36.0 |
| | 35.2 | 39.8 |
| | average 35.9 | average 38.0 |
| 4–5 | 36.4 | 42.2 |
| | 33.1 | 35.9 |
| | 38.9 | 37.6 |
| | 46.4 | 35.2 |
| | average 38.7 | average 37.7 |
| 5–6 | 36.4 | 38.8 |
| | 34.7 | 39.1 |
| | 38.9 | 40.9 |
| | 32.0 | 35.2 |
| | average 35.5 | average 38.5 |
| 6–7 | 38.0 | 43.9 |
| | 41.3 | 45.6 |
| | 42.1 | 45.8 |
| | 49.6 | 41.3 |
| | average 42.8 | average 44.2 |
| Germination (%)*** | 0.0 | 32.5 |

* Time from beginning of imbibition in Warburg flasks at 37° C.

** Each entry represents a single flask containing 50 seeds.

*** Percentages refer to germination after 2 days for the 200 seeds in each treatment. Germination in kinetin had begun by 24 hours in this experiment.

At the very high temperature GA is effective under conditions (i.e., in the presence of either kinetin or thiourea) where some germination is possible in its absence. This is in contrast to the data of table II which show that GA did not affect the mitotic activity in the nongerminating seeds. These considerations therefore favor the conclusion that the failure of GA to affect mitotic activity at 37° C (tables I and II) cannot be related to its incapacity to stimulate germination at 37° C. If so, we should expect GA to have no effect on mitosis in nongerminated seeds under conditions for which treatment with GA alone does significantly stimulate germination. The results of the next subsection will indicate that this is correct.

EFFECTS OF GA AND PHOTOMORPHOGENIC RADIATION ON MITOSIS AT 31.5 ° C: A temperature of 31.5° C permits experiments with GA and light analogous to those with kinetin and thiourea described in the first subsection and summarized in figure 1 and tables I and II. As can be seen from figure 2, germination in water is negligible at 31.5° C under continuous far-red light. However, treatment with GA stimulates germination under the same conditions of temperature and far-red lighting. Similarly, continuous exposure to red light permits germination of seeds in water. We examined 50 radicles from nongerminated seeds after 1, 2, or 3 days for each of the three treatments: red, far-red, or far-red plus GA (see table VI). In each of the three treatments, contingency tests showed there was no significant difference in the occurrence of radicles having one or more mitotic figures detected after 1, 2, or 3 days. Consequently, the data for each of the three time periods are combined within any one treatment at the bottom of table VI. There are no significant differences in mitotic activity that can be attributed to treatment with GA or to the different light conditions. These results show that GA has no direct effect on mitotic activity even at a temperature where it stimulates germination. Similarly, red and far-red light treatments, which have such different effects on germination, do not give significantly different results on mitotic activity in nongerminated seeds. After only 1 day under far-red light at 31.5° C, seeds in $10^{-5}$ M kinetin or 3 × $10^{-2}$ M thiourea gave 28 and 12% germination, respectively.

## DISCUSSION

VALIDITY OF CONCLUSIONS DRAWN FROM OBSERVATION OF MITOTIC FIGURES: In general, conclusions on the effect of any given treatment on mitosis that are drawn from numbers of mitotic figures observed may be open to any of the following criticisms: A. Detection of increased numbers of mitotic figures for a given treatment may not necessarily result from a stimulation of greater numbers of cells to undergo mitosis but rather from an effect on the time course

TABLE V

SYNERGISTIC ACTION OF GA WITH EITHER KINETIN OR THIOUREA ON GERMINATION AT 37.5° C

| SUPPLEMENT* | NO. SEEDS | GERMINATION** |
|---|---|---|
| | | (%) |
| None | 195*** | 0.0 |
| GA | 183*** | 0.0 |
| Kinetin | 453† | 8.2[A] |
| Kinetin + GA | 464† | 19.4[A] |
| Thiourea | 465† | 2.8[B] |
| Thiourea + GA | 462† | 13.6[B] |

* Concentrations as in figure 1.

** After 3 days at 37.5° C in darkness.

*** From two dishes of approximately 90 to 100 seeds each.

† From five dishes of approximately 90 to 100 seeds each.

[A, B] Pairs of figures with the same symbols significantly different from each other, P = $10^{-4}$.

of the mitotic cycle. B. Increased numbers of mitotic figures may not result from a given treatment, but may rather result from biological variability in which some organisms sampled have either a vigorous extent of cell division or are undergoing a wave of cell division at the time of fixation. C. Treatments with growth-regulating agents that seem to affect the mitotic ratio may do so by altering the growth pattern of the tissue so that cells examined after different treatments may not have come from equivalent or comparable morphological regions. The conclusions presented here are not invalidated by the first two criticisms since the pattern of deviation from randomness in the detection of mitotic figures permits the application of contingency tests on the basis of radicles having some mitosis vs. no detected mitosis. Moreover, the large numbers of individual radicles examined tend to minimize the second criticism if the total numbers of mitotic figures observed among all radicles examined in one given treatment are considered. The third criticism does not apply because all radicles examined were excised from nongerminated seeds; consequently, no total enlargement had occurred. Moreover, the data are not expressed as mitotic ratios but as numbers of mitotic figures detected per radicle.

TABLE VI

EFFECTS OF LIGHT AND GA ON MITOTIC ACTIVITY IN NONGERMINATED SEEDS AT 31.5° C

| No. of mitotic figures | Treatment → | Numbers of radicles | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Red | | | Far-red | | | Far-red + $3 \times 10^{-4}$ M GA | | |
| | Days → | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 0 | | 21 | 35 | 34 | 29 | 34 | 32 | 36 | 34 | 35 |
| 1 | | 7 | 6 | 5 | 10 | 6 | 2 | 4 | 2 | 7 |
| 2 | | 5 | 1 | ... | 3 | 4 | 2 | 1 | 1 | 2 |
| 3 | | 6 | 2 | 2 | 4 | ... | 2 | 3 | 3 | 1 |
| 4 | | 2 | 2 | ... | 1 | 1 | 3 | 2 | 2 | ... |
| 5 | | ... | ... | 1 | 1 | ... | ... | ... | 2 | ... |
| 6 | | 1 | 2 | ... | ... | ... | ... | 2 | ... | 1 |
| 7 | | ... | 1 | 2 | 1 | ... | 3 | ... | 1 | ... |
| 8 | | 2 | ... | 1 | ... | ... | ... | ... | 1 | 1 |
| 9 | | ... | ... | 1 | ... | 1 | 4 | ... | 1 | ... |
| 10 | | ... | ... | 2 | ... | ... | ... | ... | ... | ... |
| 11 | | 1 | ... | ... | ... | ... | 1 | ... | ... | 1 |
| 12 | | 1 | ... | ... | ... | 1 | 1 | ... | ... | ... |
| 13 | | ... | 1 | 1 | ... | ... | ... | ... | ... | ... |
| 14 | | ... | ... | ... | ... | ... | .. | ... | ... | ... |
| 15 | | 1 | ... | ... | ... | 2 | ... | ... | ... | ... |
| 16 | | 2 | ... | ... | ... | ... | ... | ... | ... | ... |
| 17 | | 1 | ... | ... | ... | ... | ... | ... | 1 | ... |
| 18 | | ... | ... | ... | 1 | ... | ... | ... | ... | ... |
| 19 | | ... | ... | ... | ... | ... | ... | 1 | ... | ... |
| 20 | | ... | ... | ... | ... | 1 | ... | ... | ... | 1 |
| 21 | | ... | ... | ... | ... | ... | ... | 1 | ... | ... |
| 22 | | ... | ... | 1 | ... | ... | ... | ... | 1 | 1 |
| 23 | | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 24 | | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 25 | | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 26 | | ... | ... | ... | ... | ... | ... | ... | 1 | ... |
| >26 | | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Total No. of radicles examined | | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Total No. of mitotic figures observed | | | 308 | | | 255 | | | 276 | |
| Radicles with 1 or more mitotic figures (%) | | | 40.0 | | | 36.7 | | | 30.0 | |

FURTHER EVIDENCE THAT CELL DIVISION AND CELL EXPANSION ARE INITIATED BY DIFFERENT MECHANISMS DURING GERMINATION: We previously concluded that cell division and cell expansion were initiated by different mechanisms during germination (8). If this is so, we may predict that there should be no necessary and general relation between the activity of any particular treatment on germination and its activity on mitosis in the absence of germination. Of the four germination-stimulating agents studied in this paper, only kinetin also stimulated mitosis in nongerminated seeds; GA or red light had no effect on such mitosis, and thiourea inhibited it. Thus these results amply confirm that cell division and the initiation of germination by cell expansion are controlled by different mechanisms during germination.

WHAT IS DORMANCY? Table IV shows that the effect of kinetin on breaking thermodormancy was unaccompanied by any readily discernable difference in oxygen consumption. In similar studies on the dormancy resulting from $\gamma$-irradiation of lettuce seed, it was found that GA or kinetin (which produced 15-fold or 20-fold increases in germination percentages, resp., compared to water controls) gave no difference in oxygen consumption per seed until root growth was visible (9). Similarly, although white light gave a 28-fold greater germination percentage of Grand Rapids lettuce seeds than far-red light, the fixation of $C^{14}$ from externally supplied radioactive bicarbonate into the alcohol- and water-soluble fraction was not significantly different under the two light conditions (12). Although other investigators have reported some changes in oxygen consumption per seed resulting from light treatments known to affect dormancy (5, 13, 15), our results suggest that the breaking of dormancy is not generally related to gross metabolic alterations until after root growth has begun. Long before growth begins, lettuce seeds couple energy to endergonic metabolic processes (12). Accordingly, it is not surprising that the metabolism of dormant seeds can be sufficient to sustain mitotic activity and that such activity can be modified by some growth-regulating chemicals (e.g., kinetin or thiourea). It would therefore seem that dormancy in seeds is not generally related either to a significant depression of over-all metabolism or to a block preventing cellular division. Because the term "dormancy" connotes a relatively inactive metabolism and a cessation of all processes concomitant to growth, it may be an inappropriate term for describing the physiologic state of seeds prevented from germinating by withholding morphogenic stimuli (e.g. red light, GA, etc.) under otherwise favorable conditions. These considerations lead to the conclusion that the definitive characteristic of dormancy in seeds is a subtle block that specifically prevents the initiation of cellular expansion. If thiourea generally inhibits mitosis (see p. 493), then the breaking of dormancy in potato tubers by thiourea (4) must similarly be related to an initial stimulation of growth by cellular expansion and not to cell division. Thus the definitive characteristic of dormancy in seeds may possibly be the definitive characteristic of dormancy in general.

GIBBERELLIN AND MITOSIS: Whereas previous studies showed that the action of GA in breaking lettuce seed dormancy must be attributed to an activation of cell expansion (8), our present findings further suggest that GA does not generally influence growth by directly affecting mitotic activity. It should be remembered that the mitotic activity studied here occurred in the radicle, the first organ to grow during lettuce seed germination. Gamma-irradiated wheat can provide a system for growth studies complementary to those studied here, i.e., growth can occur by cellular expansion in the absence of any cell division (10). In such a system, GA was found to give typical effects on seedling growth (10). Considerations of growth as a function of radiation dose further suggested that the increased growth induced by GA in unirradiated young wheat seedlings can be attributed largely or entirely to a direct effect on cell expansion rather than a direct effect on cell division (10). Thus the experiments performed on the two complementary systems (lettuce seeds in which cell division occurs in the absence of growth by cell expansion and irradiated wheat in which seedling growth by cell expansion occurs in the absence of cell division) suggest a single consistent theory. The growth-stimulatory effects of GA are caused solely by a direct effect on cellular expansion; thus the apparent effects on cell division are results, and not causes, of such growth stimulation (see ref. 10 for additional discussion and literature citations).

SYNERGISTIC ACTIONS INVOLVING GA ON SEED GERMINATION: Synergistic actions on lettuce seed germination from combinations of GA and kinetin (or kinetin analogues) have been reported (11, 23, 24). However, no such synergistic effect has been reported for combinations of gibberellin and thiourea, which is demonstrated here (table V). Inasmuch as kinetin and thiourea have such different over-all effects on lettuce seeds (as evidenced by their opposite effects on mitotic activity in nongerminated seeds), the fact that GA can give synergistic effects with either compound tends to weaken the suggestion of Skinner et al that gibberellin and 6-(substituted) purines "are closely related in their biochemical functions" (24). The present considerations agree with studies that showed that the actions of GA and kinetin on lettuce seed germination are separable and distinct (11).

ACTION OF KINETIN: Since the discovery of kinetin and its activity in cell division (18), it has been found to produce diverse effects, other than promotion of cell division, in several systems (see ref. 27 for review). Its effect on leaf disk expansion has been attributed to cell enlargement (6, 16). Its activity in stimulating lettuce seed germination must also be attributed to cell expansion (8). Kinetin can break dormancy resulting from $\gamma$-irradiation under

conditions where the initiation of mitosis lags several days behind the initiation of germination by cell expansion (8, 9). Consequently, this initial stimulation of germination in γ-irradiated seeds cannot possibly be related to cell division since there is no division at the time. Since kinetin has been shown to promote cell expansion in several instances, an increased cell number resulting from kinetin treatment (7, 19) does not in itself imply a primary action on cell division. Since the stimulation of cell division in nongerminated seeds occurs without over-all expansion, our studies show that kinetin can indeed act as an accelerator of cell division. That this stimulation of cell division can not be attributed to the stimulatory effect of kinetin on germination follows from the failure of the other germination-stimulators (GA, thiourea, red light) to similarly stimulate cell division in nongerminated seeds. This demonstration of a true stimulation of cell division by kinetin is in accord with the work of Skoog and his associates. These workers found that, in the presence of indoleacetic acid, kinetin treatment of tobacco pith resulted in clusters of very small cells (25). The action of kinetin in the nongerminated seeds differs from its action in tobacco pith in that no *exogenous* auxin is necessary for activity.

EFFECTS OF LIGHT: In the classic studies by the Beltsville workers (2), red and far-red light were applied to seeds as short pulses instead of the continuous light treatments used in the present studies. Since we found no difference in the mitotic activity resulting from continuous red or far-red light treatments, there should have been no differences in mitotic activity from light treatments of short duration. We chose continuous light treatment to obtain the great differences in germination percentages (fig 2), in contrast to which the lack of effect on mitosis (table VI) is all the more striking.

That the over-all effect of light on seeds differs from the over-all effects of either kinetin or thiourea is indicated by the fact that light apparently has no effect on mitotic activity whereas kinetin or thiourea do have. The effects of red light and kinetin on the initiation of germination proper are mediated by different mechanisms (11, 17). Although apparently neither red light nor GA influence mitosis in nongerminated seeds, we do not wish to imply that the effects of these two agents on germination are therefore closely related. We have previously shown that under certain conditions where lettuce seeds are unable to respond to GA they can respond to red light (11). Conversely, under certain conditions where the response to light was poor, there was a very strong response to GA (9).

From considerations analogous to those presented in the subsection on gibberellin and mitosis, it might be suggested that the effects of light on cell division during seedling growth (29) may result from more direct actions on growth by cell expansion.

EFFECTS OF THIOUREA: Although thiourea must stimulate germination via cellular expansion, it nonetheless inhibits cell division in nongerminated seeds. It also greatly inhibits mitotic activity in protruded roots (2–4 mm long) of lettuce under conditions where both treated and untreated seeds have fully germinated (Haber and Luippold, unpub.) Treatment of lettuce seeds with thiourea after γ-irradiation increased production of chromosomal aberrations in the root tips after germination (9). Thus thiourea has (at least) two different effects on the nuclear level: mitotic inhibition and (potentiation of) chromosome breakage. Inhibition of mitosis may explain the drastic inhibitory and lethal effects of thiourea on seedling growth (9, 21, 28); on inhibiting or greatly retarding cleavage of eggs and arrest of growth at the earliest developmental stages in echinoderms (1, 22); and on inhibition of regeneration in planarians (14). It may also be possible that mitotic inhibition may be related to the activity of thiourea as a goitrogenic drug, especially since goiters are characterized by a greater extent of cellular hypertrophy than cellular proliferation (31).

It is most interesting that thiourea, which inhibits growth [including that of lettuce seedlings (9, 28)] as well as mitosis in nongerminated lettuce seeds, should stimulate the initial cellular expansion in breaking dormancy of lettuce seeds and potato tubers (4). This might be partly explained by postulating that the growth-inhibitory action of thiourea can be attributed solely to inhibition of cell division. If so, then it should be relatively ineffective as a growth inhibitor in systems where mitotic activity does not significantly contribute to over-all growth as, for example, in the initial growth by cell expansion in the breaking of dormancy. In this connection, it is interesting to note that Parry found a synergistic action of thiourea and indoleacetic acid on the growth of *Avena sativa* L. seedlings (20). There was, moreover, a slight positive effect of thiourea in the absence of applied indoleacetic acid (20). This might suggest that thiourea stimulates cellular expansion in certain special cases apart from its action in breaking dormancy.

## SUMMARY

The effects of several germination-stimulating agents on mitotic activity in radicles of nongerminated lettuce seeds have been studied using conditions near the upper limits of temperatures permitting germination. Under appropriate conditions where gibberellin, kinetin, thiourea, and red (as compared with far-red) light could stimulate germination, only kinetin stimulated mitotic activity. Kinetin can be considered a true cell division factor in nongerminated lettuce seeds. Neither gibberellin nor red light had any significant effect on mitotic activity under conditions where they were later active in stimulating germination. The conditions under which thiourea inhibited mitosis were the same as those under which it later stimulated germination. The results are discussed in relation to seed germination, the general problem of dormancy,

and the actions of the germination-stimulating agents on other aspects of growth and development.

## Acknowledgments

The authors wish to express their gratitude to Dr. John Jagger for assistance in calibration of the light sources and to Drs. R. F. Kimball and Drew Schwartz for reviewing the manuscript and for their many helpful suggestions.

## Literature Cited

1. Bevelander, G. 1946. The effect of thiourea on the development of the sea urchin, *Arbacia punctulata*. Proc. Soc. Exp. Biol. Med. 61: 268–270.
2. Borthwick, H. A., S. B. Hendricks, E. H. Toole, and V. K. Toole. 1954. Action of light on lettuce-seed germination. Bot. Gaz. 115: 205–225.
3. Curcio, J. and C. C. Petty. 1951. The near infrared absorption spectrum of liquid water. Jour. Opt. Soc. Amer. 41: 302–304.
4. Denny, F. E. 1926. Hastening the sprouting of dormant potato tubers. Amer. Jour. Bot. 13: 118–125.
5. Evenari, M., G. Neumann, and S. Klein. 1955. The influence of red and infrared light on the respiration of photoblastic seeds. Physiol. Plantarum 8: 33–47.
6. Griffith, M. M. and R. D. Powell. 1959. The effect of kinetin and red light on growth and cell size. ASB Bull. 6: 24–25.
7. Guttman, R., and A. Back. 1958. Effects of kinetin on cell division in *Paramecium caudatum*. Nature 181: 852.
8. Haber, A. H. and H. J. Luippold. 1960. Separation of mechanisms initiating cell division and cell expansion in lettuce seed germination. Plant Physiol. 35: 168–173.
9. Haber, A. H. and H. J. Luippold. 1959. Dormancy resulting from gamma-irradiation of lettuce seed. Int. Jour. Radiation Biol. 1: 317–327.
10. Haber, A. H. and H. J. Luippold. 1960. Effects of gibberellin on gamma-irradiated wheat. Amer. Jour. Bot. 47: 140–144.
11. Haber, A. H. and N. E. Tolbert. 1959. Effects of gibberellic acid, kinetin, and light on the germination of lettuce seed. In: Photoperiodism and Related Phenomena in Plants and Animals. R. B. Withrow, ed. Pp. 197–206. Amer. Assoc. Advance Sci., Washington, D. C.
12. Haber, A. H. and N. E. Tolbert. 1959. Metabolism of $C^{14}$-bicarbonate, $P^{32}$-phosphate, or $S^{35}$-sulfate by lettuce seed during germination. Plant Physiol. 34: 376–380.
13. Hagen, C. E., H. A. Borthwick, and S. B. Hendricks. 1954. Oxygen consumption of lettuce seed in relation to photocontrol of germination. Bot. Gaz. 115: 360–364.
14. Jenkins, M. M. 1959. The effects of thiourea and some related compounds on regeneration in planarians. Biol. Bull. 116: 106–114.
15. Leopold, A. C. and F. S. Guernsey. 1953. Respiratory responses to red and infra-red light. Physiol. Plantarum 7: 30–40.
16. Miller, C. O. 1956. Similarity of some kinetin and red light effects. Plant Physiol. 31: 318–319.
17. Miller, C. O. 1958. The relationship of the kinetin and red-light promotions of lettuce seed germination. Plant Physiol. 33: 115–117.
18. Miller, C., F. Skoog, F. Okumura, M. von Saltza, and F. Strong. 1956. Isolation, structure, and synthesis of kinetin, a substance promoting cell division. Jour. Amer. Chem. Soc. 78: 1375–1380.
19. Orr, M. F. and B. McSwain. 1957. The effects of kinetin upon epithelium and fibroblasts in tissue culture. Cancer 10: 617–624.
20. Parry, D. W. 1952. A growth interaction between $\beta$-indolylacetic acid and thiourea (thiocarbamide). Nature 170: 1074.
21. Parry, D. W. 1956. The effects of 2,4-dichlorophenoxyacetic acid, 2,3,5-tri-iodobenzoic acid, and thiourea on the vegetative and reproductive growth of *Avena sativa*. Jour. Exp. Bot. 7: 381–386.
22. Rulon, O. 1950. The modification of developmental patterns in the sand dollar by thiourea. Physiol. Zoöl. 23: 248–257.
23. Skinner, C. G. and W. Shive. 1958. Synergistic effect of gibberellin and 6-(substituted)-purines on germination of lettuce seed. Arch. Biochem. Biophys. 74: 283–285.
24. Skinner, C. G., F. D. Talbert, and W. Shive. 1958. Effect of 6-(substituted) purines and gibberellin on the rate of seed germination. Plant Physiol. 33: 190–194.
25. Skoog, F. and C. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia Society for Exp. Biol. XI: 118–131.
26. Snedecor, G. W. 1956. Statistical Methods. Iowa State College Press, Ames. (5th ed.).
27. Strong, F. M. 1958. Topics in Microbial Chemistry: Antimycin, Coenzyme A, Kinetin, and Kinins. John Wilev & Sons, Inc., New York.
28. Thompson, R. C. and W. F. Kosar. 1939. Stimulation of germination of dormant lettuce seed by sulphur compounds. Plant Physiol. 14: 567–573.
29. Thomson, B. F. 1954. The effect of light on cell division and cell elongation in seedlings of oats and peas. Amer. Jour. Bot. 41: 326–332.
30. Toole, E. H., S. B. Hendricks, H. A. Borthwick, and V. K. Toole. 1956. Physiology of seed germination. Ann. Rev. Plant Physiol. 7: 299–324.
31. Turner, C. D. 1955. General Endocrinology. (2nd. ed.) W. B. Saunders Co., Philadelphia.
32. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques. (3rd ed.) Burgess Publishing Co., Minneapolis.
33. Withrow, R. B. and V. Elstad. 1953. Water-cooled lamp systems with refluxing aqueous filters. Plant Physiol. 28: 334–338.
34. Withrow, R. B. and L. Price. 1953. Filters for the isolation of narrow regions in the visible and near-visible spectrum. Plant Physiol. 28: 105–114.

# ACTION OF MALEIC HYDRAZIDE ON DORMANCY, CELL DIVISION, AND CELL EXPANSION[1]

ALAN H. HABER AND JOE D. WHITE[2]

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY[3], OAK RIDGE, TENNESSEE

Maleic hydrazide (MH) is of practical and theoretical interest as a growth-regulating chemical that can inhibit plant growth without causing obvious morphological abnormalities (13, 14). After recent reports of growth interactions between MH and gibberellic acid (GA) (2, 10), Brian and Hemming called attention to the many growth effects for which MH has actions opposite to those of GA (1). From studies of interactions of MH and GA on pea stem extension, these authors concluded that "MH prevents the response to GA of GA-sensitive plants" and that it is, therefore, likely that "MH inhibits growth by blocking some essential reaction at a stage preceding that where GA normally exerts its effect" (1). In these experiments, we have studied the effects of MH and compared them with those of GA on three systems: lettuce seed germination, mitosis in the absence of growth by expansion in dormant lettuce seeds (5, 7), and growth by cell expansion in the absence of mitosis in $\gamma$-irradiated wheat (6). The results show that there is no specific interaction between MH and GA either on lettuce seed germination or on the growth of wheat seedlings, and MH seems to exert direct effects on cell division but not on cell expansion during seedling growth [which is in contrast to previously studied GA effects on these same systems (6, 7)]. Similarities between effects of MH and of ionizing radiation are noted.

## MATERIALS AND METHODS

Experiments were performed with lettuce (*Lactuca sativa* L., var. Big Boston White) and wheat (*Triticum vulgare* Vill., (Triticum aestivum L.), var. Thorne). Big Boston White was used because its germination is more sensitive to MH than any of several other varieties we tested. We sowed 100 mg of lettuce seed on one piece of Whatman No. 1 filter paper moistened with 3.5 ml of solution at pH 5.7 in a covered 9 cm Petri dish. The dishes within closed copper sterilization cans (and thus in continuous darkness) were placed in a constant-temperature water bath. The criterion for germination was visual detection of radicle protrusion after 2 days at 26° C. Radicles from nongerminated seeds at 29° C were excised, fixed, and examined for mitotic figures as previously described (5, 7). Some wheat grains were given 800 kr of $\gamma$ rays at 49 kr/hr from a $Co^{60}$ source described elsewhere (3). The wheat was from the same batch used in previous studies (6) and was grown under the same controlled conditions for 12 days (with light intensity of approximately 450 ft-c during the 16-hour day). Growth was measured as length of the first leaf 12 days after sowing, at which time growth of the irradiated plants growing without mitosis was complete (6).

## RESULTS

If the suggestion of Brian and Hemming (1), that MH prevents response to GA, is generally true for gibberellin responses then we should expect GA to be less effective than other germination stimulators in relieving the dormancy imposed by MH. Accordingly, we studied the effects of germination stimulators (GA, kinetin, thiourea) in combination with germination inhibitors (MH, coumarin, 2,4-dinitrophenol, mannitol, and nicotine) on lettuce seed germination. The results given in table I show that GA and the other two stimulators seem to have the same effects relative to one another in relieving dormancy imposed by MH as in relieving dormancy imposed by the other four inhibitors. From these results it seems unlikely that there is any specific interaction between MH and GA on lettuce seed germination. This argument is valid only if the promoters stimulate germination via different mechanisms. That GA and kinetin stimulate lettuce seed germination via different mechanisms is indicated by the findings that these two compounds have different temperature ranges of activity, and that the interaction between them can be either more or less than additive, depending on experimental conditions (9). That GA, kinetin, and thiourea each have different over-all effects on seeds before germination is indicated by their different effects on mitotic activity in nongerminated seeds: no effect, stimulation, and inhibition, respectively (7).

From the original finding that dormant lettuce seeds may show mitotic activity (5), it became possible to study the effect of growth-regulating agents on such activity in the absence of growth by expansion (7). For the lettuce seeds studied in this paper, a temperature of 29° C in darkness prevented germination without completely preventing mitotic activity in the dormant seeds. Thus any effects of MH on such activity should be attributed to direct actions on cell di-

[1] Received November 5, 1959.

[2] Biology Student Trainee from Mississippi Southern College, Hattiesburg.

[3] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

TABLE I

EFFECTS OF GERMINATION-INHIBITING AND -STIMULATING CHEMICALS ON LETTUCE SEED GERMINATION

| STIMULATOR | MOLARITY OF STIMULATOR | GERMINATION %* | | | | |
|---|---|---|---|---|---|---|
| | | INHIBITOR | | | | |
| | | MALEIC HYDRAZIDE $1.5 \times 10^{-2}$ M | COUMARIN $3 \times 10^{-4}$ M | 2,4-DINITROPHENOL $2 \times 10^{-4}$ M | MANNITOL 0.25 M | NICOTINE $10^{-3}$ M |
| None | | 5 | 2 | 1 | 0 | 23 |
| GA | $10^{-5}$ | 20 | 5 | 6 | 0 | 22 |
| | $10^{-4}$ | 29 | 11 | 7 | 2 | 36 |
| | $10^{-3}$ | 32 | 14 | 7 | 4.5 | 46 |
| Kinetin | $5 \times 10^{-7}$ | 37 | 7 | 5 | 3.5 | 31 |
| | $5 \times 10^{-6}$ | 48 | 27 | 6 | 12.5 | 59 |
| | $5 \times 10^{-5}$ | 68 | 54 | 3 | 24 | 64 |
| Thiourea | $10^{-4}$ | 7 | 3 | 2 | 0.5 | 28 |
| | $10^{-3}$ | 9 | 4 | 3 | 0.5 | 25 |
| | $10^{-2}$ | 32 | 13 | 2 | 5 | 41 |

Water controls gave 72 % germination.
* Refers to 250 to 260 seeds among three replicate dishes.

vision and not to indirect effects resulting from arrested growth. The results (table II) show that the mitotic activity in such nongerminated seeds is greatly inhibited by $3 \times 10^{-3}$ M MH. This is in contrast to previous results from work with GA, which does not affect such mitotic activity (7).

Studies with the system just described, consisting of some cell division in the absence of growth by cell expansion, can be complemented by studies with a system in which growth by cell expansion occurs without cell division (6). It was previously shown that wheat grown from grain given 612 kr of $Co^{60}$ $\gamma$ rays can grow into small seedlings without any mitotic activity. (No mitotic figures were detected in root and shoot meristems fixed every day for 12 days; whereas several thousand figures were detected in similarly treated unirradiated controls.) Consequently, wheat grown and treated under exactly the same conditions but given 800 kr can be presumed to have no mitotic activity (6). The effect of MH on growth of wheat from grain given 800 kr, as well as of unirradiated wheat, is given in figure 1. Whereas the highest concentrations produce approximately 85 to 90 % inhibition in the growth of unirradiated wheat, there is no significant inhibition of the irradiated wheat. Thus these MH treatments, which can inhibit growth of the unirradiated wheat undergoing both cell division and cell expansion, cannot inhibit growth of irradiated wheat undergoing cell expansion without cell division. This might suggest that the inhibitory effect of MH on seedling growth can generally be attributed to an inhibition of cell division, a suggestion sustained by the finding that MH can inhibit cell division in the absence of growth by expansion (table II). This suggestion will be considered more critically in the Discussion section. Figure 1 shows that, with increasing MH concentra-

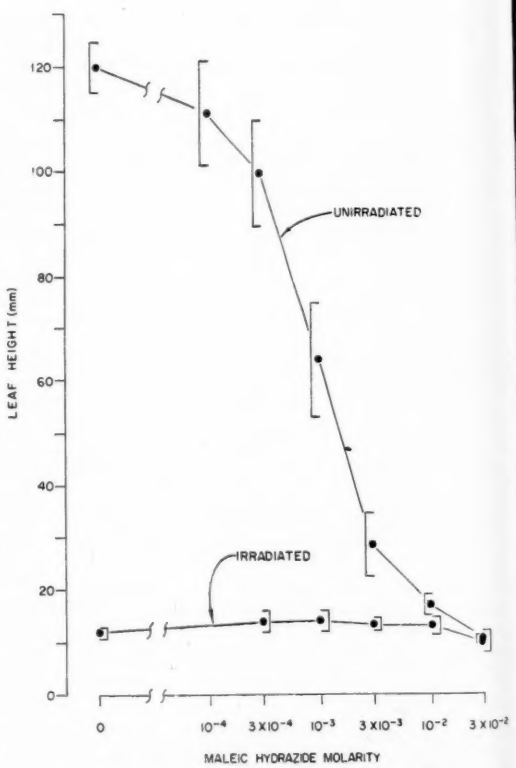



FIG. 1. Height of wheat grown from unirradiated and irradiated (800 kr) grain 12 days after sowing on solutions of MH. Brackets represent 95 % confidence limits.

TABLE II

EFFECT OF MH ON MITOTIC ACTIVITY IN DORMANT LETTUCE SEEDS AFTER 24 HOURS AT 29° C IN DARKNESS

| MOLARITY OF MH | GERMINATION* | NO. RADICLES EXAMINED | TOTAL NO. MITOTIC FIGS OBSERVED | NO. RADICLES WITH | | |
|---|---|---|---|---|---|---|
| | | | | 1 OR MORE FIGS | 3 OR MORE FIGS | 5 OR MORE FIGS |
| | (%) | | | | | |
| 0 | 0.9 | 50 | 87 | 13 | 8 | 4 |
| $3 \times 10^{-3}$ | 0.0 | 50 | 1 | 1 | 0 | 0 |

* Percentages refer to 108 seeds for each treatment.
** Germinated seed excluded.

tion, the growth curve of the unirradiated wheat appears to approach that of the irradiated wheat. If it is assumed that the sole effect of MH on wheat seedlings is to inhibit cell division, these results might suggest that the maximal inhibition by MH would correspond to a concentration that reduces mitotic activity to the extent that cell division does not contribute to growth. If so, then the high concentrations of MH would, like $\gamma$ rays, restrict growth to cell expansion. When unirradiated wheat grains were sown on solutions of $3 \times 10^{-2}$ M MH and root and shoot meristems were fixed every day for 12 days, no mitotic figures were found. This is in striking contrast to the thousands of figures observed in untreated wheat from the same batch of grain and growing under the same experimental conditions (6). In contrast to MH, GA does exert characteristic growth effects on this irradiated wheat even at doses as high as 1,225 kr (6).

Interactions of MH and GA on the growth of unirradiated wheat are given in figure 2A. GA and MH each produce their expected effects; moreover, plants treated with MH plus GA show more growth than those treated with MH alone and less growth than those treated with GA alone. The results also confirm the observations of Brian and Hemming that the effects of GA and MH are less than additive (1). It is our contention, however, that the assumption that arithmetic additivity of GA and MH effects is a necessary condition for independent action is incorrect. A more likely requirement for independent action would be that the percentage increase in growth from GA treatment should be the same in the absence of MH as in the presence of MH. The latter condition is fulfilled by the results shown in figure 2A. GA treatment gives approximately a 60 % growth increase over controls. When plants are treated with MH plus GA, the increase is also about 60 % compared to treatment with MH alone. Our conclusion that MH and GA apparently act independently (i.e., exhibit no growth interaction) will be considered more fully in the Discussion section. Independent actions on the growth of wheat seedlings is consistent with the data for lettuce seed germination (table I), which do not indicate any specific interaction of MH and GA. Independent actions would also be expected if MH inhibited seedling growth solely by cell division, since GA apparently exerts its growth-stimulatory effects in this wheat only by cell expansion (6). Therefore, we should expect that the growth response to GA of the irradiated wheat growing without mitosis should be unaffected by MH. This expectation is confirmed by the results given in figure 2B.

## DISCUSSION

Many previous investigations have led to a commonly accepted conclusion that MH is an antimitotic agent (see ref 4 and refs cited therein). Our finding that MH inhibits cell division in dormant lettuce seed contributes new information in two respects. First, this inhibition of mitosis shows that the action of MH on cell division is probably direct and cannot be attributed to arrested growth since dormant embryos do not grow. Second, this is an instance whereby MH and GA do not produce opposite effects since GA has no effect on mitosis in nongerminated lettuce seeds, even under conditions where it stimulates germination (7). The experiments with irradiated

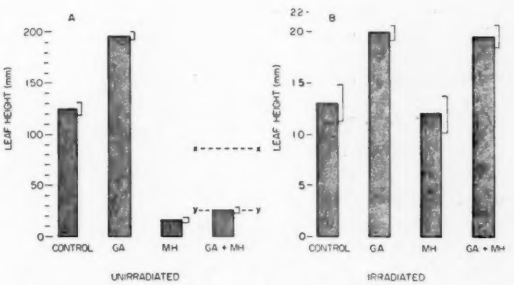



FIG. 2. Height of wheat 12 days after sowing on solutions of GA, MH, or both. Concentrations, GA, $3 \times 10^{-4}$ M; MH, $10^{-2}$ M; GA + MH, $3 \times 10^{-4}$ M GA plus $10^{-2}$ M MH. Brackets represent 95 % confidence limits. A. Wheat grown from unirradiated grain. x --- x represents height expected if the effects of MH and GA are arithmetically additive (i.e., $h_{GA} - h_{control} = h_{GA + MH} - h_{MH}$). y --- y represents height expected if the GA effect on MH-treated plants is in proportion to the GA effect in the absence of MH (i.e., $h_{GA}/h_{control} = h_{GA + MH}/h_{MH}$). B. Wheat grown from irradiated (800 kr) grain. Note change of scale.

wheat growing without cell division indicate that, in this system at least, MH has no effect on expansion. This growth of the heavily irradiated wheat by cell expansion is true growth sustained by metabolism and is not a physical artifact, since it involves a great increase in dry weight, is prevented by 2,4-dinitrophenol (Haber, unpubl.) and gives typical growth responses to GA [fig 2B; (6)]. These results both with dormant lettuce seed (where cell division occurs without growth by cell expansion) and with γ-irradiated wheat (where growth by cell expansion occurs without cell division) are consistent with the conclusion that MH inhibits cell division but has no effect on expansion (4). However, the results with irradiated wheat may not necessarily parallel the effect of MH on cell expansion in unirradiated wheat; our results do not preclude the possibility that wheat has a radiation-sensitive component of cell expansion that can be inhibited by MH. Moreover, inhibition of lettuce seed germination by MH must be an expression of a prevention of cell expansion and cannot be related to the inhibition of cell division studied here. This is because cell expansion is necessary and sufficient for breaking lettuce seed dormancy, whereas cell division is not (5, 7). Nevertheless, the fact that MH does not affect growth of the irradiated wheat which grows without cell division whereas GA does affect such a system, provides another instance whereby MH and GA do not produce opposite effects.

Since GA apparently affects normal growth of young wheat seedlings solely by stimulating cell expansion (6), the results presented in figure 2A further suggest that MH affects seedling growth solely or primarily by inhibiting cell division. Accordingly, we should expect that for different MH treatments, the GA effect per cell would be the same and would produce the same percentage increase in growth with or without MH, i.e., the effects should appear to be independent. However, failure to find a significant growth interaction in the results of figure 2A does not prove that generally there is no such interaction. Nevertheless, when some of Brian and Hemming's data are reinterpreted, they also seem to show that MH and GA can act independently on the growth rate of Meteor pea seedlings. In these plants pretreated with 0, 0.11, 0.33, or 1.0 g of MH per 100 ml, the ratios of the growth rates for plants given 10 μg GA to those given no GA were 3.3, 3.0, 3.5, and 3.8, respectively (calculated from data of fig 2 of ref 1). Thus, although the effect of MH plus GA was less than would be expected from arithmetic additivity, it was just what would be expected if GA had given the same percentage increase in growth for any MH pretreatment from 0 to 1.0 g per 100 ml. Therefore, according to our interpretation, their data also suggest that MH and GA can act independently. The finding that MH and GA apparently act independently on seedling growth has its parallel in the evidence denying a specific interaction in lettuce seed germination (table I). These considerations might also explain several other relations observed by Brian and Hemming. The greater effect of MH on tall pea as compared to dwarf peas (measured as millimeter growth/day) may have been a consequence of the greater initial height of the tall peas and their nearly fivefold greater growth rate in the absence of any chemical treatments (1). Also, their observation that "MH was slow to produce its maximum retarding effect on growth, whereas GA accelerated growth very rapidly" (1) would be expected, since direct effects on cell division should result in delayed observable effects on over-all growth, whereas direct effects on expansion should result in early observable effects.

The action of MH to induce chromosome breakage (12) might in itself conceivably contribute to growth inhibition. The work of Schwartz and Bay (15) indicates that aneuploidy and cell lethality, resulting from chromosome breakage in dividing cells, may indeed slightly reduce over-all growth. However, our previous data suggest that chromosome breakage in itself could not account for more than a few per cent of the total growth inhibition from γ irradiation (see fig 1 of ref 6). Since γ irradiation can cause reversal in the seedling height-dose curve (6) and MH does not (fig 1), we should expect that the fraction of MH-induced growth inhibition resulting from chromosome breakage would be even less than the corresponding fraction of such γ-irradiation-induced growth inhibition. Any effect of such breakage on over-all growth cannot result in aneuploidy unless the cell divides. Consequently, by using the heavily irradiated wheat, the effect of MH on cell expansion is separated from its action on aneuploidy resulting from chromosome breakage as well as from its action on mitosis.

Our results show several striking similarities between effects of MH and effects of ionizing radiation. It has long been known that chromosome breakage can result from ionizing radiation or from MH (12). Similarly, mitosis in dormant lettuce seeds can be inhibited by MH (table 2) and also, presumably, by ionizing radiation (5, 8). That grain given certain high doses of ionizing radiation can germinate and undergo limited growth without any cell division, was first suggested by Schwartz and Bay (15). This radiation effect seems to be duplicated by MH (see Results section). The growth of this wheat is extraordinarily insensitive to γ radiation in doses greater than those sufficient to prevent mitosis. By this we mean that, from 500 to 1200 kr, there is no significant effect of radiation dose on subsequent growth (6). Also MH treatment of such heavily irradiated wheat does not decrease growth any further (figs 1 and 2B). Consequently, cell expansion in these young wheat seedlings is apparently equally insensitive to γ radiation and to MH, especially since the growth curves for irradiated and unirradiated wheat seem to converge with increasing MH concentration (fig 1). That the inhibitory effects of 800 kr of γ rays and those of MH are not at all additive (figs 1 and 2B) further suggests that these two treatments affect

growth by common mechanisms. Finally, the well-known effects of MH on inducing dormancy [table I; (11)] have their counterpart in the finding that a failure of γ-irradiated lettuce seeds to germinate could be attributed to dormancy resulting from the irradiation (8). In all four of these respects in which MH effects resemble those of ionizing radiation (chromosome breakage, mitotic inhibition, relative ineffectiveness on cell expansion, and dormancy), the only one in which GA exerts effects opposite from MH is on dormancy. We have seen, however, that this cannot be attributed to any specific interaction between MH and GA (table I). Thus, treatment with GA after γ irradiation has no effect on the yield of chromosomal aberrations (8); GA does not seem to influence mitotic activity directly (7); GA, however, does stimulate growth of γ-irradiated wheat growing without mitosis [fig 2B; (6)].

This discussion favors caution in interpreting the nature of growth interactions solely from observations of over-all growth and the criterion of arithmetic additivity. None of the experiments described here bear directly on the reported activity of MH as an inhibitor of auxin action (11). However, since the primary action of MH on seedling growth seems to be inhibition of cell division, it is unlikely that MH and auxin generally and directly influence the same mechanisms that regulate growth. This last suggestion is apparently consistent with the discussion of the relation between MH and auxin presented by Leopold (11).

## Summary

MH can inhibit cell division in dormant lettuce seeds in which cell division occurs in the absence of growth by cell expansion. Concentrations of MH that inhibit normal growth of wheat seedlings by 85 to 90 % have no effect on the growth of wheat from irradiated grain, which grows without any cell division. Thus MH affects mitosis in a system where GA does not, and MH has no effect on cell expansion in a system where GA is active. When wheat is treated with a combination of MH and GA, the two chemicals apparently act independently on growth. All these findings suggest that the effect of MH on seedling growth can be largely or entirely attributed to an inhibition of cell division and not to any appreciable effect on cell expansion. MH and GA do not appear to give specific interactions on lettuce seed germination, thereby providing further evidence against the theory that MH and GA affect growth through common mechanisms. A number of similarities between effects of MH and of ionizing radiation are discussed, including the capacity to permit germination and limited seedling growth of wheat without cell division.

## Acknowledgments

The authors wish to express their gratitude to Mrs. Helen J. Luippold for her capable assistance in portions of this work and to Dr. Douglas Davidson for carefully reviewing the manuscript and for his many helpful suggestions.

## Literature Cited

1. Brian, P. W. and H. G. Hemming. 1957. The effect of maleic hydrazide on the growth response of plants to gibberellic acid. Ann. Appl. Biol. 45: 489–497.
2. Bukovac, M. J. and S. H. Wittwer. 1956. Gibberellic acid and higher plants. I. General growth responses. Quart. Bull. Mich. Agric. Exp. Sta. 39: 307–320.
3. Conger, A. D., M. L. Randolph, C. W. Sheppard, and H. J. Luippold. 1958. Quantitative relation of RBE in Tradescantia and average LET of gamma-rays, X-rays, and 1.3-, 2.5-, and 14.1-Mev fast neutrons. Radiat. Res. 9: 525–547.
4. Greulach, V. A. and J. G. Haesloop. 1954. Some effects of maleic hydrazide on internode elongation, cell enlargement, and stem anatomy. Amer. Jour. Bot. 41: 44–50.
5. Haber, A. H. and H. J. Luippold. 1960. Separation of mechanisms initiating cell division and cell expansion in lettuce seed germination. Plant Physiol. 35: 168–173.
6. Haber, A. H. and H. J. Luippold. 1960. Effects of gibberellin on gamma-irradiated wheat. Amer. Jour. Bot. 47: 140–144.
7. Haber, A. H. and H. J. Luippold. 1960. Effects of gibberellin, kinetin, thiourea, and photomorphogenic radiation on mitotic activity in dormant lettuce seed. Plant Physiol. 35: 486–494.
8. Haber, A. H. and H. J. Luippold. 1959. Dormancy resulting from gamma-irradiation of lettuce seed. Int. Jour. Radiation Biol. 1: 317–327.
9. Haber, A. H. and N. E. Tolbert. 1959. Effects of gibberellic acid, kinetin, and light on the germination of lettuce seed. In: Photoperiodism and Related Phenomena in Plants and Animals, R. B. Withrow, ed. AAAS, Washington, D. C. Pp. 197–206.
10. Kato, J. 1956. Effect of gibberellin on elongation, water uptake, and respiration of pea-stem sections. Science 123: 1132.
11. Leopold, A. C. 1955. Auxins and Plant Growth. University of California Press, Berkeley.
12. McLeish, J. 1952. The action of maleic hydrazide in Vicia. Heredity 6 (suppl.): 125–147.
13. Naylor, A. W. and E. A. Davis. 1950. Maleic hydrazide as a plant growth inhibitor. Bot. Gaz. 112: 112–126.
14. Schoene, D. L. and O. L. Hoffmann. 1949. Maleic hydrazide, a unique growth regulant. Science 109: 588–590
15. Schwartz, D. and C. E. Bay. 1956. Further studies on the reversal in the seedling height dose curve at very high levels of ionizing radiations. Amer. Naturalist 90: 323–327.

# EFFECT OF SODIUM AND POTASSIUM SALTS ON GROWTH AND OXALATE CONTENT OF HALOGETON

M. COBURN WILLIAMS [1, 2]

DEPARTMENT OF BOTANY, UTAH STATE UNIVERSITY, LOGAN, UTAH

*Halogeton glomeratus* (M. Bieb.) C. A. Mey. is a poisonous annual weed which has become established on approximately 10.5 million acres of the western range. It is found primarily in Utah, Nevada, Idaho, and Wyoming but occurs also in California, Oregon, Colorado, and Montana. In addition to causing direct losses from poisoning of sheep, halogeton seriously reduces the number of acres suitable for grazing over broad areas formerly used for winter sheep range.

Fleming, Miller, and Vawter (6) found in 1942 that the toxic substance in halogeton was oxalic acid, and further work by Dye (5) established that this poison occurred in its toxic form primarily as a soluble salt. The leaves of a vigorous halogeton plant usually contain in excess of 30 percent soluble oxalates (dry weight) during the late summer and early autumn. In addition, about three or four percent of the dry weight consists of insoluble oxalates believed to be primarily salts of Ca or Mg.

Either Na or K would be required in rather large quantities to maintain the cation-anion balance within the plant and to supply cations for each molecule of oxalate produced. Experimental evidence has shown that halogeton accumulates large quantities of these univalent cations and that Na is absorbed much more readily than K. Dye (5) found a leaf-seed-sepal fraction of halogeton to contain 14.52 percent Na vs. 0.73 percent K and Beath et al (2), in whole plant analysis, found Na to comprise 28.6 percent of the dry weight vs. 2.6 percent K. Dye (5) also found that the leaf-seed-sepal fraction contained more than 5 percent Cl. These data indicate that soluble oxalates are likely to occur primarily as Na salts and that NaCl is the principal salt form absorbed on the desert.

The use of Na in plant nutrition has been extensively studied. This element has been reported essential as a micronutrient for bladder saltbush, *Atriplex vesicaria*, Heward (3), and for the blue-green alga, *Anabaena cylindrica* (1). Sodium has never been shown to completely replace K, although Na may in many instances produce both increases in yield and dry weight. An increased growth with NaCl was demonstrated in Salicornia by Van Eijk (12). Lehr (9) obtained significantly greater yields in spinach with $NaNO_3$ than $Ca(NO_3)_2$. Spinach absorbed more K if equal amounts of Na and K were available, but Na reduced the requirement for K and improved yields even when K was not limiting.

The work described herein deals with the effect of the addition of Na and K salts to nutrient cultures upon oxalate concentration in halogeton leaves and upon plant size, dry weight, and vigor. Preliminary work showed that halogeton leaves were the only valid index that could be used to determine the effect of the experimental salts on oxalate content, since other plant tissue (stems, flowers, seeds) did not contain large quantities of soluble oxalates.

## MATERIALS AND METHODS

Halogeton seedlings were germinated in the greenhouse in washed white sand. Two days after seedling emergence the sand was moistened with a dilute (1:4) Hoagland's nutrient solution (7). The seedlings remained in the sand for 10 days until the first leaves were well formed then transferred to one-gallon polyethylene freezer containers containing full-strength Hoagland's nutrient solution plus the experimental salts. The seedlings were inserted into the tops of the lids and held in place by polyethylene ringlets. In addition to the full sunlight of the greenhouse, two 200-watt lights were placed above the halogeton to insure a 16-hour day. The minimum candle-power received throughout the test area during the period of supplemental illumination was 450 ft-c. If halogeton receives less than 400 ft-c of supplemental light after July 15 in the greenhouse, flowering, which was undesirable in this experiment, occurs. Polyethylene capillary tubing was inserted into the center hole in the lid and aeration under pressure of 5 psi was provided continuously by a 1/6 HP pump. The tops of the lids were covered with black plastic tape to exclude light and the containers were lightproofed with aluminum foil.

The basic nutrient solution in which the control plants were grown was prepared according to Hoagland (7) with deionized water and reagent grade chemicals. The total Na contamination present in the basic solution was 1.2 to 1.8 ppm, as determined by flame spectrophotometric analysis (13). Initial Cl contamination averaged 0.18 ppm.

The treatments consisted of adding reagent grade NaCl, $NaNO_3$, and KCl separately to Hoagland's nutrient solution at concentrations of 0.0, 0.001 *N*, and 0.01 *N*. Sodium chloride was also added at a concentration of 0.1 *N*. Two additional levels of Na were provided by nutrient solutions containing

[1] Revised manuscript received November 9, 1959.

[2] Plant Physiologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, in cooperation with the Utah Agricultural Experiment Station, Logan.

0.002 *N* and 0.02 *N* $Na_2SO_4$. Six containers holding two plants each were used per treatment.

The plants were grown for 7 weeks in the solution culture. A complete change of solution was made every 2 weeks. Twenty milligrams of $FeSO_4 \cdot 7\ H_2O$ was added twice weekly. Approximately 2 months after germination, and just prior to harvest, the plants were measured for width of lateral spread. In the cruciform type of growth which occurs in halogeton, i.e., one central stem and four laterals, the most rapid growth during the first 8 to 10 weeks occurs in the lateral branches. The measurement shown in table I indicates the mean spread in centimeters from the tip of one lateral to the tip of that lateral directly opposite. The plants were harvested, sectioned into stems, roots, and leaves, and oven-dried at 65° C for 24 hours. Following a determination of the dry weight of both the leaves and the entire plant, the leaves were analyzed for K, Na, and soluble and insoluble oxalates.

A similar but modified experiment was later conducted in a growth chamber. The plants received 15 hours of continuous illumination at 1,100 ft-c supplied by banks of fluorescent lights. A daytime temperature of 80° F and a nighttime temperature of 68° were maintained. Sodium nitrate and 0.001 *N* NaCl treatments were omitted because of space limitations, and the solutions were changed and buffered biweekly to pH 6.7 with a mixture of $K_2HPO_4$ and $KH_2PO_4$.

Leaves were collected monthly from April to October from halogeton on a plot adjacent to highway U.S. 30S, 11 miles west of Snowville, Utah. The leaves were washed, oven-dried, and analyzed for oxalates according to Dye (5). A fraction was digested in a 1 : 1 mixture of $HNO_3$ and $HClO_4$, evaporated to near dryness, brought to a desired volume in deionized water, and analyzed for Na and K in a flame spectrophotometer (10). Analyses for Na, K, and oxalates were conducted in the same manner for the greenhouse and growth-chamber plants.

## Results

Greenhouse. At the time of harvest the plants grown in nutrient solutions to which Na salts had been added were large and succulent. As indicated in tables I and II, adding Na to the basic nutrient solution resulted in the production of halogeton significantly larger in physical size, of higher dry weight, and with a higher concentration of oxalates than both the controls and plants grown in nutrient solutions containing KCl. Another Na effect observed was that of leaf size and succulence. Basal leaves obtained at the juncture of the main stem and the branches were found to average 1.5 × 12 mm in plants which received Na vs. 1 × 7 mm in those of the controls or the 0.001 *N* KCl treatment. Plants which received 0.01 *N* KCl produced leaves 1.2 × 9 mm. Maximum succulence and physical size were obtained in the 0.1 *N* NaCl treatment in which the basal leaves averaged 2 × 16 mm. The smaller, slightly-curled leaves of the halogeton of the KCl treatments and the control solutions appeared to be in a state of semi-wilt throughout the 7-week growth period. This condition was apparent at all times and was particularly evident during the afternoons when the temperature in the greenhouse was usually between 90 to 100° F. Wilting was not evident in plants grown in the growth chamber where the temperature did not

Table I

Dry Weight and Lateral Branch Expansion of Halogeton Grown 7 Weeks in Nutrient Cultures Containing NaCl, KCl, $NaNO_3$, and $Na_2SO_4$

| Treatment | Width of lateral branches | | Dry wt of leaves | | Dry wt of entire plant | |
|---|---|---|---|---|---|---|
| | Gh* | Gc* | Gh | Gc | Gh | Gc |
| | *cm* | *cm* | *gm* | *gm* | *gm* | *gm* |
| Control** | 15.9 | 9.06 | 0.120 | 0.073 | 0.285 | 0.125 |
| 0.001 *N* KCl | 16.4 | 16.00 | 0.140 | 0.247 | 0.331 | 0.452 |
| 0.01 *N* KCl | 25.3 | 19.10 | 0.252 | 0.330 | 0.610 | 0.636 |
| 0.001 *N* NaCl | 22.8 | ... | 0.300 | ... | 0.800 | ... |
| 0.01 *N* NaCl | 26.5 | 24.40 | 0.400 | 0.370 | 0.980 | 0.747 |
| 0.1 *N* NaCl | 37.5 | 33.60 | 0.750 | 0.777 | 1.695 | 1.521 |
| 0.001 *N* $NaNO_3$ | 27.6 | ... | 0.434 | ... | 0.924 | ... |
| 0.01 *N* $NaNO_3$ | 27.7 | ... | 0.356 | ... | 1.020 | ... |
| 0.002 *N* $Na_2SO_4$ | 28.1 | 18.50 | 0.377 | 0.253 | 0.907 | 0.473 |
| 0.02 *N* $Na_2SO_4$ | 31.7 | 19.50 | 0.437 | 0.353 | 1.017 | 0.669 |
| LSD (0.05) | 2.51 | 2.2 | 0.05 | 0.02 | 0.02 | 0.06 |
| LSD (0.01) | 3.33 | 2.9 | 0.06 | 0.03 | 0.03 | 0.08 |

* Gh–Greenhouse; Gc–Growth chamber

** Hoagland's nutrient solution

exceed 80° F. The curled, wilted condition was never observed in plants which received Na.

The differences in dry weights of the leaves indicated in table I primarily reflect differences in the number of leaves produced. This is also true in the comparisons of the whole plant dry weight. Plants which received Na tended to produce numerous additional side branches on the central and four cruciform branches, while plants which received no Na produced very few or none.

The soluble oxalate content of halogeton was greatly increased by adding Na to the nutrient solution. Plants grown in NaCl had a much higher oxalate content than plants which received any other salt. Adding KCl to the nutrient solution did not result in the production of plants with a high leaf oxalate content, although the content was significantly greater than the oxalate content of the controls. The soluble oxalate content of the KCl and control series was lower than that obtained with any level of sodium salt, regardless of the anion present. Growth was generally dependent upon the concentration of Na in solution, but oxalate formation was stimulated more by NaCl than by $Na_2SO_4$ or $NaNO_3$, despite the availability of equivalent or greater concentrations of Na.

When Na was limiting and present only in contamination concentrations, K was absorbed readily by halogeton (table II). The me K absorbed by the plants which received 1 and 10 me K as KCl was equal to or higher than the me Na absorbed by halogeton supplied a similar quantity of Na as $NaNO_3$ or NaCl. Adding Na to the nutrient solution resulted in a marked decrease in the absorption of K. Although the concentration of K in the basic solution was 6 me, the me of Na absorbed was nearly double K when as little as 1 me Na was added. These data indicate that when Na is available, halogeton will absorb this cation in preference to K, but when Na is limiting, K will be substituted at least in quantity, even though it is unable to substitute for Na biochemically.

Growth Chamber. Although seedlings which died were replaced during the first 3 days after initiation into the culture solution, only 6 of the 12 initial control plants and 7 of 12 in each of the KCl treatments survived. Plants which were grown in solutions containing a Na salt were more vigorous and total losses during the first 3 days did not exceed three plants in any treatment. Total losses from the 3rd day until harvest did not exceed two plants in the Na treatments.

At harvest, the control plants were much smaller than those in the greenhouse experiment. This difference in size could in part be attributed to lower Na contamination of the nutrient solution, which resulted in a more pronounced Na deficiency. The plants grown in KCl had a slightly higher dry weight than those of the greenhouse experiment, but plants grown in $Na_2SO_4$ solutions were much smaller. Plants in the $Na_2SO_4$ treatment grew vigorously for about five weeks, after which growth became markedly retarded. Two plants died just prior to harvest in the 7th week. The retarded growth and mortalities suggested that Cl became limiting despite the occurrence of this anion as a trace element in the culture solution. Adding 5 ppm Cl as $CaCl_2$ has prevented a reoccurrence of this problem in subsequent experiments in which a Cl form of the salt was not used. The more vigorous growth observed in the greenhouse may have been the result of sufficient Cl contamination to meet the minimum requirement for this species.

The maximum Cl contents of the reagent grades $Na_2SO_4$ and $NaNO_3$ were 0.002 % and 0.0008 %, respectively. The concentration of Cl in the nutrient

Table II

Sodium, Potassium, and Oxalate Content of Halogeton Leaves When Plants Were Grown in Nutrient Cultures Containing KCl, NaCl, $NaNO_3$, and $Na_2SO_4$

| Treatment | Greenhouse | | | | Growth chamber | | | |
|---|---|---|---|---|---|---|---|---|
| | me/g dry wt | | | | me/g dry wt | | | |
| | Na | K | Soluble oxalates | Total oxalates | Na | K | Soluble oxalates | Total oxalates |
| Control* | 0.11 | 3.78 | 1.09 | 1.53 | 0.02 | 2.10 | 2.17 | 2.74 |
| 0.001 *N* KCl | 0.21 | 4.00 | 1.54 | 2.30 | 0.02 | 4.35 | 2.91 | 3.14 |
| 0.01 *N* KCl | 0.15 | 4.48 | 2.02 | 2.62 | 0.03 | 4.54 | 3.00 | 3.29 |
| 0.001 *N* NaCl | 2.80 | 1.38 | 3.13 | 3.26 | ... | ... | ... | ... |
| 0.01 *N* NaCl | 3.80 | 1.36 | 3.66 | 3.66 | 3.44 | 1.79 | 4.34 | 4.34 |
| 0.1 *N* NaCl | 6.20 | 1.80 | 3.90 | 3.90 | 5.30 | 1.27 | 4.44 | 4.44 |
| 0.001 *N* $NaNO_3$ | 3.18 | 1.83 | 2.19 | 2.66 | ... | ... | ... | ... |
| 0.01 *N* $NaNO_3$ | 4.70 | 2.00 | 2.62 | 3.19 | ... | ... | ... | ... |
| 0.002 *N* $Na_2SO_4$ | 3.75 | 1.50 | 2.52 | 2.82 | 3.30 | 1.75 | 3.52 | 3.89 |
| 0.02 *N* $Na_2SO_4$ | 5.20 | 1.19 | 2.77 | 3.30 | 4.15 | 1.19 | 3.77 | 3.98 |

* Hoagland's nutrient solution

solution could have been increased a maximum of 0.028 ppm by $Na_2SO_4$ and 0.0068 ppm by $NaNO_3$ at the highest concentration used. The Cl content of the basic solution consistently analyzed 0.35 ppm or above. Since the control plants and those grown in solutions containing $Na_2SO_4$ received essentially equal concentrations of Cl, the difference in growth and oxalate content must have been the result of adding Na. A deficiency of Cl could not have been the primary factor in the poor growth and low oxalate content of the control, since the Cl deficiency was not evident in $Na_2SO_4$ plants for several weeks while retarded growth of the controls was evident immediately after the seedlings were placed in the control solution. Further, the addition of 35 and 355 ppm Cl as KCl did not compensate for the absence of Na in the greenhouse.

The leaves of halogeton grown in the buffered solution in the growth chamber contained more oxalates than plants grown in the greenhouse (table I). The controls showed the most notable increase with a rise in soluble oxalates from 4.9 to 9.8 percent. Plants supplemented with NaCl were markedly larger and contained considerably more oxalates than plants grown in any other treatment.

Buffering was found to be necessary, since unbuffered solutions gave pH values between 3.0 and 3.5 after 2 weeks. When the solutions were buffered, the 2-week readings ranged from 6.0 in the controls to between 4.0 and 5.6 in those containing KCl, $Na_2SO_4$, and 0.01 *N* NaCl. The final pH of the 0.1 *N* NaCl solution was 3.7.

All plants which did not receive NaCl contained both soluble and insoluble oxalates, whereas only soluble oxalates were present in leaves of halogeton grown in 0.01 *N* and 0.1 *N* NaCl. Complete absence of insoluble oxalates has never been observed in desert-grown plants.

ANALYSIS OF DESERT PLANTS. In a monthly analysis of oxalates in halogeton fractions containing a mixture of leaves, seeds, and sepals, Dye (5) had shown that the quantity of soluble oxalates remained fairly constant during the growing season and increased during the late fall and early winter. Dye's analysis utilized fractions which would normally be consumed by sheep and did not take into consideration the relative amounts of each plant tissue present. In the experiment discussed herein, only the leaves were analyzed so that the results could be compared with greenhouse results.

A leaf and a leaf-seed-sepal sample obtained during August were compared for soluble oxalates. The leaves contained 6.16 me soluble oxalates per gram of dry tissue, whereas the leaf-seed-sepal fraction contained 4.85 me. It was apparent, therefore, that the presence of seeds and sepals diluted the overall oxalate concentration. Seeds contain only about 0.4 to 1.7 me soluble oxalates when mature; therefore, the analysis of the leaf-seed-sepal fraction would tend to vary according to the ratio of the three plant segments (14).

When the first desert collections were made, only cotyledons containing small amounts of soluble oxalates were present. By May the leaves contained more than 3.8 me soluble oxalates; the concentration continued to rise steadily during the summer. In September the oxalate content rose sharply. At this time, the longer leaves present during the summer had begun to disappear and the leaves collected were a smaller type, which subtended the flowers. These were particularly high in oxalates; they are the leaves which occasion the sharp rise in toxicity during the autumn, even when mixed with the less toxic seeds and bracts. The maximum quantity of both oxalates and Na occurred in September (table III).

## DISCUSSION

Sodium appears to be an essential element in the nutrition of halogeton. Whether this element can properly be classed as a micro- or macronutrient must await further study under more controlled conditions. Since contamination in the greenhouse usually resulted in a Na concentration of 6 to 8 ppm in the basic solution between biweekly changes, Na was usually present in micronutrient quantities in the control and KCl treatments. Nevertheless, the striking results obtained with very high concentrations of Na would suggest that optimum growth and oxalate content occur only when this element is present in substantial quantities in the soil or nutrient culture.

The Na in halogeton leaves appears to be used primarily to form salts of oxalic acid. The use of Na in this metabolic process is particularly evident in table III. The total Na present in May, June, and August would have neutralized only 96.8 to 98.7 percent of the oxalates formed. The Na concentration was adequate for the oxalate concentration in September and was in excess in July and October. The K present in the leaf tissue could have neutralized only 18 to 19 percent of the oxalic acid in May, June, July, and October; 29.5 percent in August, and only 11.9 percent during September, the month when solu-

TABLE III

OXALATE, SODIUM, AND POTASSIUM CONTENT OF HALOGETON LEAVES COLLECTED NEAR SNOWVILLE, UTAH

| DATE OF COLLECTION | SOLUBLE OXALATES | TOTAL OXALATES | NA | K |
|---|---|---|---|---|
| | *Me/g dry weight* | | | |
| April 15* | 0.30 | 0.50 | ... | ... |
| May 15 | 3.82 | 4.85 | 3.70 | 0.71 |
| June 15 | 5.31 | 6.27 | 5.15 | 0.96 |
| July 15 | 5.62 | 6.22 | 7.00 | 1.03 |
| August 15 | 6.16 | 7.19 | 6.10 | 1.82 |
| September 15 | 7.69 | 8.61 | 7.84 | 0.92 |
| October 15** | 6.66 | 7.64 | 7.69 | 1.30 |

* Only cotyledons present
** Plants dead

ble oxalate content was at a maximum. Had all the K present been utilized in oxalate formation, approximately 81 to 88 percent of the soluble oxalates present would have required Na.

The work of Dunne (4), Ulrich (11), and Jacobson and Ordin (8) has clearly demonstrated that excess of cations over inorganic anions was balanced by organic acid production within the plant. This metabolic process in halogeton results primarily from excess absorption of Na. The data in table II indicate, however, that Na is more effectively used than K in the production of soluble oxalate salts. When equal concentrations of K and Na were available as KCl and NaCl in the basic solution, only 66.9 percent of the total leaf oxalates occurred as soluble salts in the 0.001 *N* KCl treatment. No oxalates were insoluble in the 0.01 *N* NaCl series while only 77 percent of the oxalates were water soluble in the 0.01 *N* KCl treatment. The absence of Ca and Mg oxalates was partially due to the Cl ion, since insoluble oxalates occurred in the leaves of plants which were grown in solutions containing equal or greater amounts of Na as $NaNO_3$ or $Na_2SO_4$. The production of plants containing only soluble oxalates was probably the result of a balanced nutrient solution containing an optimum concentration of NaCl. Optimum conditions are not necessarily associated with the availability of NaCl alone, since insoluble oxalates were found to occur in the leaves of halogeton plants collected within a few feet of the Great Salt Lake. These plants were rooted in soil which was extremely high in NaCl.

A significantly-increased succulence of leaves and stems was consistently observed when Na was added to the basic solution. The work of Van Eijk (12) on Salicornia indicated that, while Na increased growth, Cl was primarily responsible for succulence. Maximum leaf succulence in halogeton was obtained when 0.1 *N* NaCl was added to the solution, but no apparent differences were observed between equivalent amounts of Na, regardless of the anion. The semi-wilt of the leaves in plants receiving 35.5 and 355 ppm Cl as KCl would indicate that Na is the more effective of the two ions in promoting succulence.

The role of Cl in halogeton nutrition is somewhat less clear than that of Na. Halogeton apparently requires a concentration of 5 to 10 ppm Cl in nutrient solution to prevent deficiency symptoms in plants during a 7-week growth period.

Meeuse and Campbell (10) demonstrated that both Cl and nitrate inhibit oxalic acid oxidase and thus cause an accumulation of oxalates. In Halogeton Cl is more effective in this respect, since $NaNO_3$ in solution was less effective than both $Na_2SO_4$ and NaCl in increasing the quantity of soluble oxalates in leaves. The failure of KCl to produce concentrations of oxalates similar to those produced by NaCl indicates that an interaction between Na and Cl occurs which promotes greater organic acid metabolism.

It is doubtful that any of the symptoms of Na deficiency discussed would ever be encountered under natural conditions. The data do indicate, however, that where the soil is high in NaCl, halogeton will tend to be exceptionally vigorous and high in soluble oxalates.

## Summary

Halogeton absorbed large quantities of Na. This element may be absorbed as a number of salt forms, but NaCl appears to be the principal salt absorbed in the desert. The leaves of both desert and greenhouse plants contained a maximum of 6.1 to 7.8 me Na (dry weight basis) during the growing season. Under natural conditions, the major portion of the Na absorbed is metabolized as sodium oxalate.

Halogeton which received no added Na in its nutrition exhibited poor growth and vigor. Leaf oxalate content, growth, and succulence were greatly increased when Na as NaCl was added to the basic solution.

Halogeton which received no added Na substituted K in concentration in the leaves. When Na was available in at least 0.001 *N* concentration, the plant absorbed more of this cation than K. Potassium did not completely substitute for Na in promoting growth and vigor or in the formation of soluble oxalates.

Plants which were grown in Na alone were slightly more vigorous and somewhat larger than those which were grown in solutions containing KCl. Greater quantities of soluble oxalates occurred in plants which received Na.

Although no optimum concentration for Cl in the basic solution was established, the data suggest that the Cl requirement of halogeton is greater than that which would be supplied by most nutrient cultures now recommended for hydroponic research.

A 0.1 *N* concentration of NaCl in the nutrient solution was most effective in promoting growth, vigor, physical size, and abundant oxalate formation. When halogeton was grown in solutions containing 0.01 *N* NaCl or above, only soluble oxalates were formed.

Sodium was essential for vigorous growth of halogeton.

## Literature Cited

1. Allen, M. B., and D. I. Arnon. 1955. Studies on nitrogen-fixing blue-green algae. II. The sodium requirement of *Anabaena cylindrica*. Physiol. Plantarum 8: 653–60.
2. Beath, O. A., C. S. Gilbert, H. F. Eppson, and Irene Rosenfeld. 1953. Poisonous plants and livestock poisoning. Wyo. Agr. Exp. Sta. Bull. 324.
3. Brownell, P. F., and J. G. Wood. 1957. Sodium as an essential micronutrient element for *Atriplex vesicaria*, Heward. Nature 179: 635–36.
4. Dunne, T. C. 1932. Plant buffer systems in relation to the absorption of bases by plants. Hilgardia 7: 207–234.

5. DYE, W. B. 1956. Chemical studies on *Halogeton glomeratus*. Weeds 4: 55–60.
6. FLEMING, C. E., M. R. MILLER, and L. R. VAWTER. 1943–1950. Project 22. Poisonous range plants–*Halogeton glomeratus*. Nev. Agr. Exp. Sta. Ann. Reports.
7. HOAGLAND, D. R., and D. I. ARNON. 1950. Water culture method for growing plants without soil. Cal. Agr. Exp. Sta. Circ. 347.
8. JACOBSON, L., and L. ORDIN. 1954. Organic acid metabolism and ion absorption in roots. Plant Physiol. 29: 70–5.
9. LEHR, J. J. 1949. Exploratory pot experiments on sensitiveness of different crops to sodium: A. Spinach. Plant and Soil 2: 37–48.
10. MEEUSE, B. J., and J. M. CAMPBELL. 1958. An inhibitor of oxalic acid oxidase in beet juice. Talk, given before the American Society of Plant Physiologists, Western Section, Logan, Utah. June 17–19.
11. ULRICH, A. 1941. Metabolism of non-volatile organic acids in excised barley roots as related to cation-anion balance during salt accumulation. Amer. Jour. Bot. 28: 526–537.
12. VAN EIJK, M. 1939. Analyse der Wirkung des NaCl auf die Entwicklung, Sukkulenz, und Transpiration bei *Salicornia herbacea* sowie Untersuchungen über den Einfluss der Salzaufnahme auf die Wurzelatmung bei *Aster tripolium*. Rec. trav. bot. néerl. 36: 559–657.
13. WILLARD, H. H., L. L. MERRITT, JR., and J. A. DEAN. 1951. Instrumental Methods of Analysis. Pp. 77–83. D. Van Nostrand Co., Inc., New York.
14. WILLIAMS, M. C. 1958. Biochemical and physiological properties of brown and black seeds of *Halogeton glomeratus*. Proceedings Sixteenth Western Weed Control Conference, Spokane, Wash. Pp. 51–55.

# UTILIZATION OF EXOGENOUS SUGARS FOR BIOSYNTHESIS OF CARBOHYDRATES IN GERMINATING POLLEN[1, 2]

GIAN KESSLER[3], DAVID S. FEINGOLD, AND W. Z. HASSID

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA

It is known that pollen germination is enhanced by supplying exogenous carbohydrate; however, little information is available concerning the fate of the carbohydrate in the pollen. O'Kelley (10) showed that when pollen is germinated in $C^{14}$-labeled D-glucose, D-fructose, or sucrose, $C^{14}$-labeled $CO_2$ is formed, indicating that the pollen metabolizes exogenous carbohydrate. Further evidence for the utilization of exogenous carbohydrate was presented by other workers (1, 7, 13), who demonstrated synthesis of starch by pollen germinated in various sugar solutions. A more detailed study of the in vitro utilization of exogenous carbohydrate by germinating pollen is presented in this paper, showing that the carbohydrate is transformed to other complex saccharides in addition to starch.

## MATERIALS AND METHODS

Uniformly $C^{14}$-labeled sucrose (74 μc/μmole) and uniformly $C^{14}$-labeled fructose (30 μc/μmole) were kindly furnished by Dr. E. W. Putman. Prior to use the sugars were purified by two-dimensional chromatography. After eluting the sugars from the paper, residual impurities, originating presumably from the chromatographic procedure, were eliminated by circular chromatography on filter paper disks which had been treated with 1 *N* acetic acid at 100° C. Omitting the latter purification step resulted in a marked inhibition of germination.

Laminaran, laminarobiose, laminarotriose, and laminarotetraose were a gift from Dr. W. J. Whelan of the Lister Institute, London. The laminarodextrin series was prepared by heating a 4 per cent solution of soluble laminaran in 0.5 *N* HCl at 100° C for 20 minutes. The solution of the partially hydrolyzed polysaccharide contained D-glucose and a homologous series of oligosaccharides ranging in degree of polymerization from two to greater than seven. Maltodextrins were obtained by heating a 4 per cent solution of soluble starch in 0.5 *N* HCl at 100° C for 5 minutes.

Emulsin (β-glycosidase) was purchased from the Nutritional Biochemicals Co. This enzyme preparation was used at a final concentration of 1 per cent in 0.01 M sodium acetate, pH 5.0. The enzyme preparation was devoid of α-glucosidase activity under the conditions of the test. The enzymatic hydrolysis with emulsin was carried out at 37° C over a period of two to six hours.

A lyophilized preparation of a specific β-1,3-D-glucanase (S 176A—*Rhizopus arrhizus* QM 1032) (12) was kindly supplied by Dr. E. T. Reese, Quartermaster Research and Development Center, Natick, Mass. The preparation was used as an 8 per cent

[1] Received November 17, 1959.
[2] This investigation was supported in part by a research grant (No. A–1418) from the U. S. Public Health Service, National Institute of Health, and by a research contract with the U. S. Atomic Energy Commission.
[3] Fellow of the "Schweizerische Stiftung fuer Stipendien auf dem Gebiete der Chemie".

solution in 0.05 *N* sodium acetate pH 4.8. Incubations were carried out in the presence of toluene at 37° C for 6 and 30 hours.

Crude centrifuged saliva was used as a source of $\alpha$-amylase. Mixtures consisting of equal volumes of the $\alpha$-amylase solution, 0.5 M sodium acetate and the individual sugar solutions were incubated at 37° C for 6 hours.

Liquid invertase concentrate was obtained from the British Drug Houses Ltd., England. The sugar solutions (20 µl) were mixed with 20 µl of 0.1 M sodium acetate and 10 µl of invertase solution, and incubated at 30° C for 6 hours.

Glass capillary tubes of 1.2 to 1.5 mm diameter were used as reaction vessels (11) in all reactions conducted in volumes of 100 µl or less.

The total carbohydrate content of pollen samples, which had been dried to constant weight in vacuum at 40° C over NaOH, was determined by the anthrone method (4) and calculated as glucose.

Germination in vitro: Pollens of the species *Lapageria rosea* and *Impatiens oliveri* were selected for this study. The material was kindly provided by Mr. P. Hutchinson, of the Botanical Garden of the University of California. In preliminary tests the molarity of sucrose solutions that would produce optimal growth over a period of 16 hours was determined for each species. Based on these results a substrate concentration of 0.15 molar was used throughout the investigation.

Series I of germination experiments were carried out with pollen in 0.15 M solutions of uniformly $C^{14}$-labeled D-fructose. Approximately 10 µmoles (300 µc) of the purified radioactive D-fructose was diluted to the specified concentration and transferred to a watch glass (approximately 65 µl per watch glass). Pollen then was gently brushed off the anthers of freshly harvested flowers and approximately 1 mg of pollen grains was added to the sugar solution. The watch glass was placed in a moist chamber, and the pollen grains were allowed to germinate for 16 hours at room temperature (25° C). At the end of the incubation period the pollen was examined microscopically to determine the per cent germination and the extent of the pollen tube growth. The pollen was then aggregated in the center of the watch glass by imparting a swirling motion to the contents, and the initial substrate was gradually displaced by repeated addition of portions of 0.15 M nonradioactive D-fructose, followed by removal of the liquid with a micropipette. The washed samples were transferred into 1 ml of boiling 95 per cent ethanol and the suspension was boiled for one minute. Subsequently the pollen was extracted repeatedly by suspension in 0.5 ml portions of hot 50 per cent ethanol and centrifugation until the supernatant liquid was free of radioactivity. Prior to analysis the residual insoluble material and the combined extracts were concentrated to dryness in a vacuum desiccator over KOH at room temperature.

In series II of germination experiments fresh pollen samples were first incubated in 0.15 M nonradioactive sucrose solution. At the end of three hours the initial substrate was displaced by 0.15 M D-mannitol. The washed pollen was resuspended in 60 µl of 0.15 M D-mannitol solution containing 0.14 µmoles (10.0 µc) of labeled sucrose. The incubations were then allowed to proceed for another 16 hours at room temperature. Subsequent steps for microscopic examination and separation of the metabolic products were identical with those described in the first series of germination experiments.

Separation and Identification of Reaction Products: Paper electrophoretic separation of borate complexes of carbohydrates was carried out on strips of oxalic acid-washed Whatman No. 1 filter paper in an apparatus similar to that described by Crestfield and Allen (2), using 0.1 M sodium tetraborate, pH 9.2, as buffer solution.

Paper chromatography of soluble metabolic products (hexoses, oligosaccharides, noncarbohydrate compounds) was performed by radial development on disks of Whatman No. 1 filter paper with *n*-propanol-ethyl acetate-water (7:2:1) at 37° C. In addition, mono- and disaccharides were chromatographed two-dimensionally on Whatman No. 1 paper using water-saturated phenol in the first dimension and butanol-acetic acid-water (52:13:35) in the second. Sugars were detected with *p*-anisidine phosphate spray reagent (1 g of *p*-anisidine phosphate in 140 ml of 70% alcohol).

Radioactive substances on paper were located by means of radioautography, using Eastman No-Screen X-ray film, and counted on paper with a Tracerlab rate meter. Under the conditions employed 1 µc of radioactivity adsorbed on Whatman No. 1 filter paper was equivalent to 90,000 cpm.

Partial acid hydrolysis of insoluble pollen material was carried out according to the method described by Feingold et al (5). The samples were transferred as an aqueous suspension to a glass tube sealed at one end and taken to dryness under reduced pressure. Approximately 0.5 ml of concentrated HCl saturated with HCl gas at −18° C was added, the tube sealed and immediately immersed in a 23° C water bath. After 10 minutes the contents of the tube were diluted with 10 volumes of water and the HCl was removed in a vacuum desiccator over KOH.

Exhaustive hydrolysis of the carbohydrate material was achieved by extending the hydrolysis time to 30 minutes. Otherwise all the manipulations were performed as described for partial hydrolysis.

In cases where chromatographic analysis indicated that the partial hydrolyzate contained more than one series of radioactive oligosaccharides, a separation of the isomers with an identical degree of polymerization was achieved by paper electrophoresis in borate buffer. After eluting the separated compounds from the paper the aqueous sugar-borate complexes were concentrated under reduced pressure, applied to the center of a Whatman No. 1 filter paper disk, and subjected to radial chromatography in an acid solvent (*n*-butanol-acetic acid-water 52:13:35). This proc-

ess dissociated the sugar-borate complexes and separated the oligosaccharides from the residual borate salts. The somewhat diffuse bands of the borate-free oligosaccharides were subsequently eluted and rechromatographed as previously described.

Radioactive mono- and oligosaccharides obtained from the alcohol extracts or partial acid hydrolyzates were identified by cochromatography and coelectrophoresis with authentic sugars. Oligosaccharides were further identified by partial as well as total hydrolysis with appropriate enzymes. Invertase was used to establish the presence of $\beta$-D-fructofuranosyl moieties, emulsin to detect $\beta$-D-glucopyranosyl residues, and salivary amylase to degrade $\alpha$-1,4-D-glucans.

HISTOLOGICAL TECHNIQUES: Starch grains in the growing pollen-tubes were detected by staining with $I_2$-KI. Callose was made visible with resorcin blue prepared as described by Mueller-Stoll and Lerch (9).

## RESULTS

GERMINATION AND TUBE GROWTH: Lapageria and Impatiens pollen germinated equally well in solutions of D-fructose or sucrose. However, tubes produced by Lapageria pollen grains germinated in D-fructose solution were somewhat shorter than those produced in sucrose solution. Identical results were obtained with radioactive and unlabeled D-fructose, indicating that there was no visible deleterious effect of the radioactivity upon germination and tube growth.

When germinating Lapageria pollen grains were transferred from 0.15 M nonradioactive sucrose solution to 0.15 M D-mannitol containing 0.14 μmoles of radioactive sucrose, further pollen-tube growth was completely inhibited. However, since a number of labeled products formed, it was clear that metabolic processes other than tube growth continued. Inasmuch as D-mannitol failed to produce any inhibitory effect, no corresponding experiment with $C^{14}$-labeled sucrose was performed with Impatiens pollen.

DRY WEIGHT AND CARBOHYDRATE CONTENT OF LAPAGERIA POLLEN: The average weight loss on drying of Lapageria pollen was 9 per cent. Its total carbohydrate content, calculated as glucose, was 26 per cent of dry weight.

SEPARATION AND IDENTIFICATION OF LABELED METABOLIC PRODUCTS: Chromatographic analysis of the 50 per cent ethanol extracts after the incubation period revealed in each case the presence of a number of labeled compounds. In addition to the original carbohydrate substrate, several newly formed radioactive carbohydrate compounds were isolated and identified by chromatographic, electrophoretic, and specific enzymatic procedures. The resulting metabolic carbohydrate products produced when Lapageria pollen was supplied with D-fructose and sucrose are presented in table I.

Identical soluble carbohydrate compounds which were formed in Impatiens pollen when germinated in D-fructose solution were identified by the same procedures. Furthermore, other chromatographically mobile substances from which acid hydrolysis failed to release chromatographically detectable monosaccharides, were present in the ethanol extracts. The chemical structure of these metabolic products has not been investigated; the products will be referred to as noncarbohydrate substances.

In all germination experiments the insoluble material remaining after the ethanol extraction was also radioactive. In the experiments with D-fructose-germinated pollen (Lapageria and Impatiens) partial acid hydrolysis of the insoluble radioactive product yielded D-glucose, a trace of D-fructose, a complex mixture of oligosaccharides, and chromatographically immobile material. The oligosaccharides were separated and found to consist of two homologous series: A. The laminarodextrin series ($\beta$-1,3-D-oligoglucosides) having a degree of polymerization ranging from two to eight, and B. The maltodextrin series

TABLE I

METABOLIC PRODUCTS OF LAPAGERIA ROSEA POLLEN GERMINATED IN SOLUTIONS OF $C^{14}$-LABELED D-FRUCTOSE AND SUCROSE

| GERMINATION SUBSTRATE | % METABOLIC PRODUCTS* | | | | | |
|---|---|---|---|---|---|---|
| | D-FRUCTOSE | D-GLUCOSE | SUCROSE | MALTOSE | SOLUBLE NONCARBOHYDRATE MATERIAL | INSOLUBLE MATERIAL |
| D-Fructose** | Substrate | 3.9 | 66.5 | 0.2 | 12.7 | 16.6† |
| Sucrose*** | 24.4 | 67.0 | Substrate | ... | 4.4 | 4.2†† |

* Calculated as proportion of radioactivity present in metabolic products other than substrate.

** 1.10 mg of fresh *Lapageria rosea* pollen germinated for 16 hours in 0.06 ml of 0.15 M radioactive D-fructose solution. 1.4 μmoles = 0.26 mg of D-fructose (26 % dry wt of pollen) were converted to other metabolic products.

*** 0.95 mg of fresh *Lapageria rosea* pollen germinated for 3 hours in 0.15 M sucrose solution, followed by incubation for 16 hours in 0.06 ml of 0.15 M D-mannitol solution containing 0.14 μmoles of radioactive sucrose. 0.06 μmoles = 0.02 mg of radioactive sucrose were converted to other metabolic products.

† $\beta$-1,3-D-glucan, starch, and insoluble noncarbohydrate material.

†† $\beta$-1,3-D-glucan, insoluble noncarbohydrate material.

($\alpha$-1,4-D-oligoglucosides) of degree of polymerization ranging from two to four. Members of the laminarodextrin series of a polymerization degree greater than eight, and of the maltodextrin series of polymerization degree greater than four were not mobile under the chromatographic conditions employed.

The fact that partially hydrolyzed insoluble material from the pollens of Lapageria and Impatiens produced a laminarodextrin and maltodextrin series of oligosaccharides indicates the presence in the pollen of these plants of $\beta$-1,3-D-glucan and starch.

The radioactive oligosaccharides obtained by partial acid hydrolysis of the Lapageria pollen grown on D-mannitol solution and supplemented with radioactive sucrose were found to consist of laminarodextrins only. Had other oligosaccharides been present, the experimental conditions would have permitted detection of approximately 5 per cent.

When Lapageria pollen grains were allowed to germinate in $C^{14}$-labeled D-fructose solution, 66.5 per cent of the radioactive products formed was recovered as sucrose, which was labeled in both the D-glucosyl and the D-fructosyl moieties. Free radioactive D-glucose was present only in minor amounts. The presence of a minute quantity of maltose was also demonstrated. Soluble noncarbohydrate compounds accounted for 12.7 per cent of the total incorporated radioactivity.

Insoluble radioactive material accounted for 16.6 per cent of the total radioactive metabolic products. Approximately 90 per cent of the latter was shown by exhaustive hydrolysis in hydrochloric acid to consist of D-glucose, the remaining 10 per cent being chromatographically immobile material. Upon partial hydrolysis of the same insoluble product, about 75 per cent of the oligosaccharides released were members of the laminarodextrin series. Maltodextrins accounted for the other 25 percent.

For the D-fructose incubation experiments with Impatiens a detailed evaluation of the distribution of the labeled compounds formed, as described for Lapageria, was not made. However, a difference was observed in the relative quantities of laminarodextrins and maltodextrins in the partial hydrolyzates of the insoluble material. In the partial hydrolyzate of the insoluble material from Impatiens pollen the maltodextrin to laminarodextrin ratio was of the order of ten to one, which is in marked contrast to the one to three ratio found in the case of Lapageria. Histological examination of germinated pollen corroborated these findings. Starch grains were present in Impatiens but could not be detected in Lapageria pollen.

$\beta$-1,3-D-Glucanase Hydrolysis of Germinated Lapageria Pollen: Microscopic examination of Lapageria pollen which had been germinated in D-fructose solution and stained with resorcin blue revealed the presence of callose in the pollen-tube walls as well as of callose inclusions in the cytoplasm of the pollen-tubes. After incubation with $\beta$-1,3-D-glucanase for a period of six hours a marked decrease of the color intensity of the callose-staining areas had occurred. Upon protracted treatment with the enzyme (30 hrs), resorcin blue failed to give a positive reaction for callose. This indicates that the blue-staining material is a $\beta$-1,3-D-glucan. In addition, the enzyme treatment effected a change in the texture of the pollen tubes, causing a progressive loss of rigidity and eventual collapse of the cell walls.

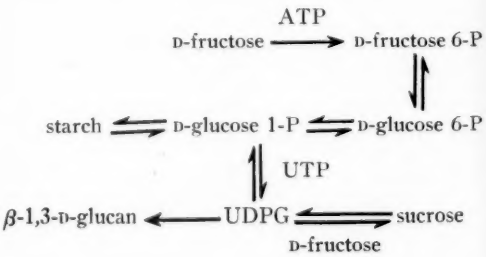
When Lapageria pollen was germinated in radioactive D-fructose solution and exhaustively extracted with 50 per cent ethanol and then treated with $\beta$-1,3-D-glucanase for 3 hours, three labeled, chromatographically mobile substances were obtained. The major product of the enzymatic hydrolysis was D-glucose; the presence of a smaller quantity of laminarotetraose could also be demonstrated. These results indicate the presence of radioactive $\beta$-1,3-D-glucan. In addition, a trace of an unidentified substance was recovered which had the chromatographic mobility of a disaccharide in the solvent system used.

## Discussion

Germinating pollen is able to transform to various metabolic products a quantity of exogenous D-fructose approximately equal to the carbohydrate initially present in the pollen, indicating that these transformations represent major metabolic processes.

Although soluble carbohydrates were the main products of metabolic transformations, germinating pollen was also found to be capable of utilizing the externally supplied sugar for the synthesis of insoluble polysaccharides. Under the experimental conditions only two radioactive polysaccharides, starch and $\beta$-1,3-D-glucan, were shown to be present; no evidence was obtained for the synthesis of cellulose or pectin, although these materials are considered to be constituents of the cell wall of pollen-tubes (9).

The enzymatic steps whereby external hexose was converted to the various carbohydrate metabolic products found in the pollen were not investigated. It is reasonable to assume, however, that the hexose is transformed via metabolic pathways which are known to operate in higher plants[4] (6):




[4] The following abbreviations are used: ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; UDPG, uridine diphosphate D-glucose.

When pollen was allowed to germinate in D-mannitol, β-1,3-D-glucan was formed from externally supplied sucrose although no tube elongation occurred after adding the labeled substrate. The pollen was therefore still metabolically active and capable of converting sucrose to polysaccharide at a time when tube elongation was completely inhibited by D-mannitol.

Hellmers and Machlis (7) have shown that pollen germinated in water depletes its internal starch reserve during germination; when exogenous soluble carbohydrate is supplied to such depleted pollen, starch is again formed. Since the D-mannitol-germinated pollen was supplied with about 50 times less substrate (sucrose) than the D-fructose-germinated pollen, lack of starch formation could conceivably have been due to the insufficient quantity of exogenous sucrose available. Presumably the β-1,3-D-glucan synthesizing enzymatic system is still active under these conditions. It is also conceivable that the starch-forming enzyme system is inactivated by the presence of D-mannitol, which might be expected to be an inhibitor of some metabolic activities since it caused complete suppression of tube growth (10).

The presence of histologically-defined callose in pollen-tubes has long been known (3); however, no information concerning its chemical structure has been available. Recently it was demonstrated by methylation analysis that callose from *Vitis vinifera* sieve tubes is a β-1,3-D-glucan (8). The finding that pollen tube callose is hydrolyzed by a specific β-1,3-D-glucanase indicates that it is similar in chemical structure to sieve tube callose. Furthermore, the formation of radioactive β-1,3-D-glucan shows that exogenous hexose is readily transformed to callose by pollen during germination.

## Summary

A study has been made of the utilization of $C^{14}$-labeled D-fructose and sucrose by germinating pollen of *Lapageria rosea* and *Impatiens oliveri*.

Sucrose was shown to be the main carbohydrate produced by the *Lapageria rosea* pollen germinated in $C^{14}$-labeled D-fructose solution. Two radioactive polysaccharides, starch and β-1,3-D-glucan, were also synthesized by the pollen.

The same metabolic products were obtained when *Impatiens oliveri* pollen was germinated in $C^{14}$-labeled D-fructose solution.

When Lapageria pollen which had been germinated in nonradioactive sucrose was transferred to a solution containing D-mannitol and $C^{14}$-labeled sucrose, pollen tube growth ceased. Under these conditions the formation of β-1,3-D-glucan, but not starch, occurred.

The fact that the material in the pollen tubes which gives a positive callose stain with resorcin blue is degraded by a specific β-1,3-D-glucanase indicates that pollen tube callose is a β-1,3-D-glucan.

## Literature Cited

1. Brink, R. A. 1924. The physiology of pollen I–IV. Amer. Jour. Bot. 11: 218–228; 283–294; 351–364; 417–436.
2. Crestfield, A. M. and F. W. Allen. 1955. Improved apparatus for zone electrophoresis. Anal. Chem. 27: 422–423.
3. Eschrich, W. 1956. Kallose. Protoplasma 47: 487–530.
4. Fairbairn, N. J. 1953. Modified anthrone reagent. Chemistry and Industry: P. 86.
5. Feingold, D. S., E. F. Neufeld, and W. Z. Hassid. 1958. Synthesis of a β-1,3-linked glucan by extracts of *Phaseolus aureus* seedlings. Jour. Biol. Chem. 233: 783–788.
6. Hassid, W. Z., E. F. Neufeld, and D. S. Feingold. 1959. Sugar nucleotides in the interconversion of carbohydrates in higher plants. Proc. Nat. Acad. Sci., U. S. 45: 905–915.
7. Hellmers, H. and L. Machlis. 1956. Exogenous substrate utilization and fermentation by the pollen of *Pinus ponderosa*. Plant Physiol. 31: 284–289.
8. Kessler, G. 1958. Zur Charakterisierung der Siebroehrenkallose. Ber. schweiz. bot. Ges. 68: 5–43.
9. Mueller-Stoll, W. R. and G. Lerch. 1957. Ueber Nachweis, Entstehung und Eigenschaften der Kallosebildungen in Pollenschlaeuchen. Flora 144: 297–334.
10. O'Kelley, J. C. 1955. External carbohydrates in growth and respiration of pollen tubes in vitro. Amer. Jour. Bot. 42: 322–327.
11. Porter, L. W. and N. Hoban. 1954. Ultramicrotechnique for enzymatic hydrolysis of sugars prior to chromatogram analysis. Anal. Chem. 26: 1846–1848.
12. Reese, E. T. and M. Mandels. 1959. β-1,3-D-glucanases in fungi. Can. Jour. Microbiol. 5: 173–185.
13. Tischler, G. 1917. Pollenbiologische Studien. Zeits. Bot. 9: 417–488.

# A ROLE FOR METHIONINE IN DIVISION OF CHLORELLA VULGARIS[1,2]

ALEX SHRIFT

DIVISION OF BIOLOGY, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

A key metabolic role can be ascribed to methionine on the basis of many demonstrable biochemical functions, even though these reactions have not all been proven to exist within one organism. Methionine is a constituent of proteins, (although there are instances in which it is reported absent (3)); it also is a source of methyl groups for a number of cellular components, undoubtedly through the intermediate. S-adenosylmethionine, the actual methyl donor (9). Of the many transmethylations that are known a few seem to be more directly linked than others to plant cell division. The incorporation of the methyl group into lignin (6, 7) and into pectin (21, 25, 26) indicates a need for methionine in cell wall formation. There is also evidence from work with animals that the methyl group can be used to a limited degree for purine and pyrimidine synthesis (16, 17). In microorganisms such a use has not been found (13), although S-adenosylmethionine can act as methyl donor to several non-naturally occurring purines (23). In plants, transmethylation from methionine is known to occur during the synthesis of several plant alkaloids (4, 5, 8, 12, 33) which as a chemical class show many resemblances to the purines and pyrimidines, but a specific connection between these reactions and plant nucleic acid synthesis is unknown. Other sulfur-containing metabolites, notably sulfhydryl compounds, are recognized as playing a significant, although speculative (34, 35) role in the division of cells in general; recently data have been presented which show sulfur to be necessary for cells of *Chlorella ellipsoidea* to divide (14, 15). Similar evidence for methionine, however, is sparse.

The idea that methionine may be intimately associated with division, stems from the observation that selenomethionine, a structural, competitive analogue of methionine, will prevent cells of *Chlorella vulgaris* from dividing but will allow them to maintain other cellular activities that lead to growth into giant cells (31). Analysis of these giant cells has revealed that there are increases in the rate of respiration, dry weight, and protein, and that the newly formed proteins lack methionine but contain other sulfur amino acids derived from sulfate (32). The ability of selenomethionine to replace methionine in several biological and biochemical systems (10, 11, 18, 36) suggests that in the uncoupled *C. vulgaris* cell there exist processes that can operate despite the substitution. The selective uncoupling effect, however, strongly implies that methionine participates in a specific, irreplaceable manner in the division mechanism of the *C. vulgaris* cell.

This report is one of a series designed to characterize more fully the relation of methionine to division. The experiments described here were performed to see if additional methionine analogues could uncouple growth from division and if the response to selenomethionine occurred in organisms other than *C. vulgaris*.

## MATERIALS AND METHODS

The strain of *Chlorella vulgaris* used, the composition and preparation of media, preparation of inocula, and counting technique have been described earlier (30); cultural environment such as light intensity, temperature, and rate of flow and composition of the gas mixture were as noted in a later paper (32).

*Chlorella pyrenoidosa* (ATCC No. 7516) was grown in medium of identical composition and under the same conditions as for *Chlorella vulgaris*. The medium designed by Sager and Granick (24) was used for the growth of *Chlamydomonas reinhardi* (obtained from Dr. Ruth Sager, Department of Zoology, Columbia University, New York City); the other cultural conditions were identical with those for *Chlorella vulgaris*.

*Escherichia coli* (ATCC No. 6522) was grown in a liquid medium based on that of Anderson (1): $NH_4Cl$, 0.0187 M; $Na_2HPO_4$, 0.0422 M; $KH_2PO_4$, 0.022 M; $MgCl_2 \cdot 6H_2O$, 0.0017 M; $K_2SO_4$, 0.00031 M; dextrose, 0.0022 M. Ammonium and phosphate salts were added as one stock solution. Dextrose and $MgCl_2$ were autoclaved separately and added later. Aliquots of the stock solutions were pipetted into Pyrex tubes, 85 × 15 mm, matched to fit the Klett Summersen colorimeter, and diluted to 5.0 ml. Filter No. 42 (blue) was used to measure optical densities. Inoculum cells, taken from 14 to 16 hour old cultures, were centrifuged and washed three times with double distilled sterile water. The cells were diluted to an optical density of about 0.40, and a 0.1 ml aliquot was used for inoculum. Duplicate tubes were incubated at 37° C on a roller-tube wheel that rotated at 5 rpm. Hourly readings were taken during the exponential phase of growth; readings were taken less frequently thereafter.

[1] Received November 18, 1959.

[2] This investigation was supported in part by a National Science Foundation grant, G–2833. Presented May 3, 1958, at the annual meeting of the northeastern section of the American Society of Plant Physiologists, Yale University, New Haven, Conn.

*Torulopsis utilis* (obtained from Dr. Dean B. Cowie, Carnegie Institute of Washington) was grown in the medium described by Olson and Johnson (20). Inoculum cells were pregrown for about 16 hours in low glucose (6 mg/5 ml) as recommended by Dr. Cowie in order to eliminate a long lag on transfer to new medium. The cells were harvested, washed, and diluted to a Klett reading of about 300. A 0.2 ml aliquot was used for inoculum. Conditions of growth were otherwise identical with those described for *E. coli*.

Most of the analogues were purchased from Nutritional Biochemicals. Methoxinine was obtained from Dr. J. P. English, American Cyanamid Co., Stamford, Conn., and methionine sulfoximine from Dr. L. Reiner, Wallace and Tiernan Products, Belleville, N. J.

## Results and Discussion

Influence of Methionine Analogues on Growth of Chlorella Vulgaris: Table I lists the analogues that were tested, and figures 1 to 4 illustrate the growth responses to each analogue. Ethionine and methionine sulfoximine caused marked growth inhibitions, whereas the other four analogues were relatively non-inhibitory. Uncoupling of growth from division was not observed.

The effect of ethionine was evident at as low a concentration as $2 \times 10^{-6}$ M (fig 1). The immediate consequence was a failure of the cells either to grow or divide; with progressive increments in the concentration, the duration of this phase was correspondingly prolonged. A second phase could be recognized during which the rate of division increased steadily to a maximum exponential rate which, however, was lower than that of the control culture.

It seems unlikely that the resumption of divisions at each ethionine level was due to a selection of resistant mutants, for the following reasons. The approximate number of mutants can be calculated by extrapolation of the slope of the exponential phase of growth to zero time if one assumes that the mutants divided at the same, unchanged rate from the time of inoculation. The calculated numbers for each concentration of the analogue, given in table II, are improbably high, and furthermore, one would have to assume a continuous series of resistant mutants, those with the lowest resistance being present in the largest numbers.

Table I

Structures of Methionine Analogues Tested on Chlorella Vulgaris

| | |
|---|---|
| Selenomethionine | $CH_3$-Se-$CH_2CH_2CHNH_2COOH$ |
| Methoxinine | $CH_3$-O- |
| Ethionine | $CH_3CH_2$-S- |
| Methionine sulfoxide | $CH_3$-S(=O)- |
| Methionine sulfoximine | $CH_3$-S(=O)(=N)- |
| Norleucine | $CH_3$-$CH_2$- |
| Norvaline | $CH_3$- |

A second feasible explanation for the renewed divisions, a non-cellular, spontaneous decomposition of the analogue under the experimental growth conditions of long duration, was checked by incubation for 239 hours before inoculation, of medium that contained $1 \times 10^{-4}$ M ethionine. This level ordinarily resulted in a lag that lasted about 150 hours. Pretreatment reduced the lag to about 75 hours (fig 1). Some change, therefore, must have occurred in the medium to reduce the toxicity of the ethionine. The simultaneous existence or development of a cellular detoxification mechanism is not excluded, however, since in vivo transformation of ethionine by yeast (29) and in vitro transethylation by yeast enzymes (22) are known.

The growth responses to graded levels of methionine sulfoximine (fig 2) differed from those observed with ethionine. At each concentration the initial effect was a slower rate of division rather than a complete halt as with ethionine. There ensued a rather abrupt decline in the division rate which then rose continuously until a maximum exponential rate was attained. This maximum rate was lower than that of the control. That these growth responses may

Table II

Calculated Numbers of Chlorella Vulgaris Mutants (Derived from fig 1), in Cells ($2 \times 10^5$/ml) Inoculated into Graded Concentrations of Ethionine

| Molar conc ethionine | Slope of exponential phase | Duration of lag (hrs) | Calculated mutants/ml at time of inoculation | Calculated % mutants at time of inoculation |
|---|---|---|---|---|
| 0 | 0.084 | 23 | ... | ... |
| $2 \times 10^{-6}$ | 0.067 | 18 | 62,050 | 31.0 |
| $8 \times 10^{-6}$ | 0.076 | 56 | 2,840 | 1.42 |
| $3 \times 10^{-5}$ | 0.057 | 110 | 354 | 0.177 |
| $1 \times 10^{-4}$ | 0.050 | 146 | 142 | 0.071 |
| $5 \times 10^{-4}$ | 0.036 | 210 | 107 | 0.054 |

Influence of methionine analogues on the growth of Chlorella vulgaris.

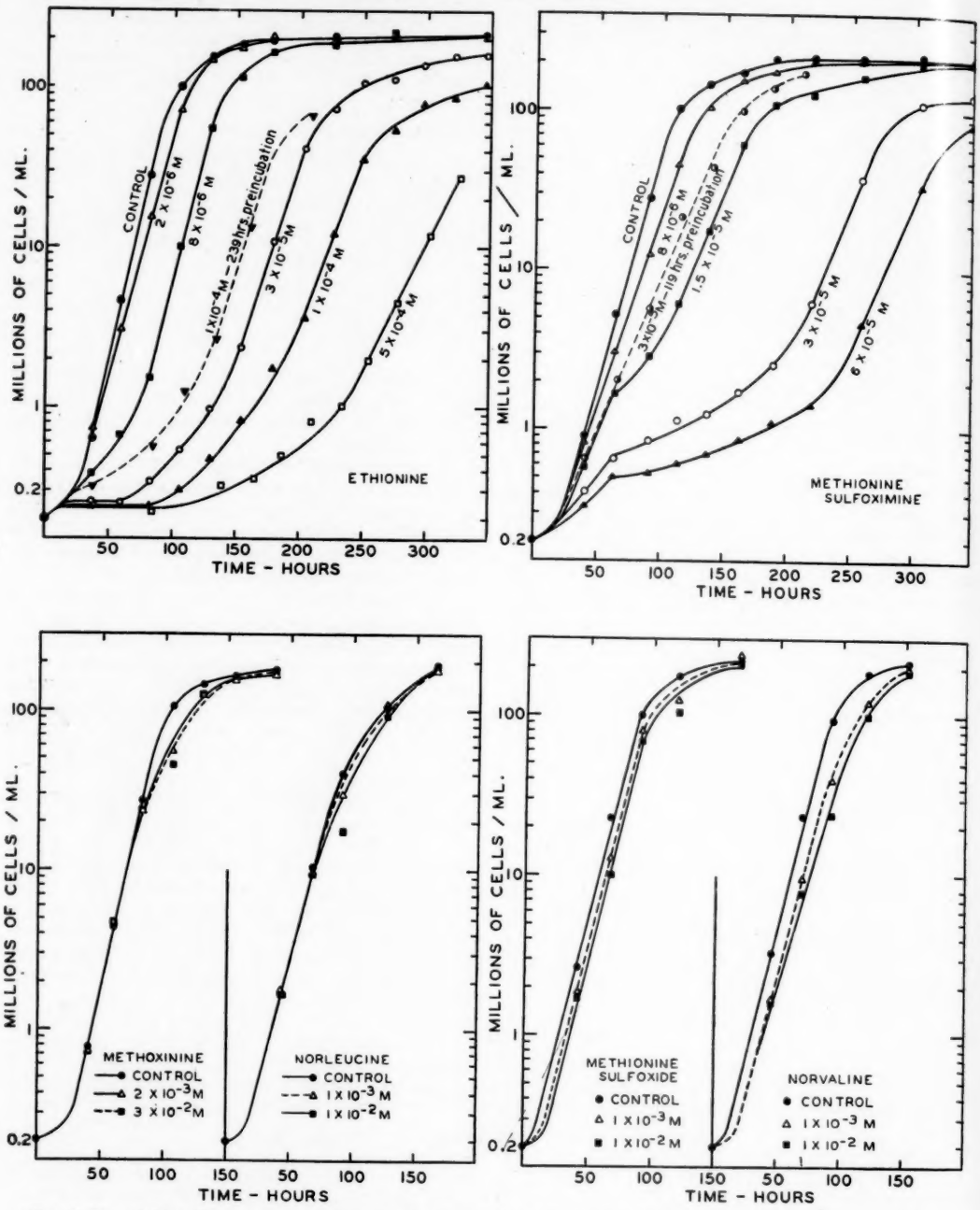



FIG. 1 (*top, left*). Ethionine. Broken line shows growth in medium incubated 239 hours before inoculation.

FIG. 2 (*top, right*). Methionine sulfoximine. Broken line shows growth in medium incubated 119 hours before inoculation.

FIG. 3 (*bottom, left*). Methoxinine and norleucine.

FIG. 4 (*bottom, right*). Methionine sulfoxide and norvaline.

have been due to alterations in the medium was investigated. Preincubation for 119 hours, of medium that contained $3 \times 10^{-5}$ M of the compound, indeed resulted in a changed medium (fig 2), but the rate of growth was inexplicably much higher than that in a culture of comparable age that had been inoculated at the very outset.

Effects by other analogues were apparent but only at the relatively high concentrations of $10^{-3}$ to $10^{-2}$ M. Methoxinine and norleucine (fig 3) had no influence on the initial lags and exponential rates, but growth in these cultures began to decelerate sooner than the controls. Chlorosis developed and was most severe in the presence of methoxinine. Slight increases in the lag phase were caused by methionine sulfoxide and norvaline; the latter depressed the growth rate slightly (fig 4).

Ethionine and methionine sulfoximine appear to have entered the cells readily, to judge from the patterns of growth inhibition; the development of chlorotic cells in the presence of methoxinine and norleucine is indicative of their absorption, even though the latter is unavailable as a nitrogen source for this species (*2*). $S^{35}$-methionine sulfoxide has been shown to penetrate *Chlorella pyrenoidosa* and its radioactivity to spread into other soluble compounds and into proteins in small amounts, but it was unable to serve as a sulfur source for growth (*27*). This compound will also enter oat tissues and yield its methyl group to form pectin, protopectin, and S-methyl methionine (*26*), but the extent of its penetration into *Chlorella vulgaris* is unknown. Without more specific absorption and biochemical data it is difficult to assess the failure of the six analogues to uncouple growth from division. The results do, however, emphasize the biological specificity of selenomethionine, which structure, only slightly changed from that of methionine, is nevertheless able to profoundly disrupt one major cellular process, division, without a comparable effect upon cell growth.

Influence of Selenomethionine on Growth of Other Organisms: Uncoupling of growth from division was not observed. Of the four microorganisms studied, *Chlamydomonas reinhardi* was the most sensitive and showed an increase in lag in the concentration range $5 \times 10^{-7}$ to $1 \times 10^{-5}$ M. At higher concentrations growth was completely inhibited during the incubation period of the experiments (fig 5). Cultures that resumed exponential divisions appeared to be normal insofar as could be judged from their green appearance. At the two highest concentrations of selenomethionine that permitted exponential divisions, $1 \times 10^{-6}$ and $1 \times 10^{-5}$ M, calculations show that the number of resistant cells in the inoculum of $3.0 \times 10^{5}$ cells/ml would have been 18,950 and 4,780, respectively. As with ethionine, there seems to be a graded series of resistants. These data do

Influence of selenomethionine on the growth of four microorganisms.

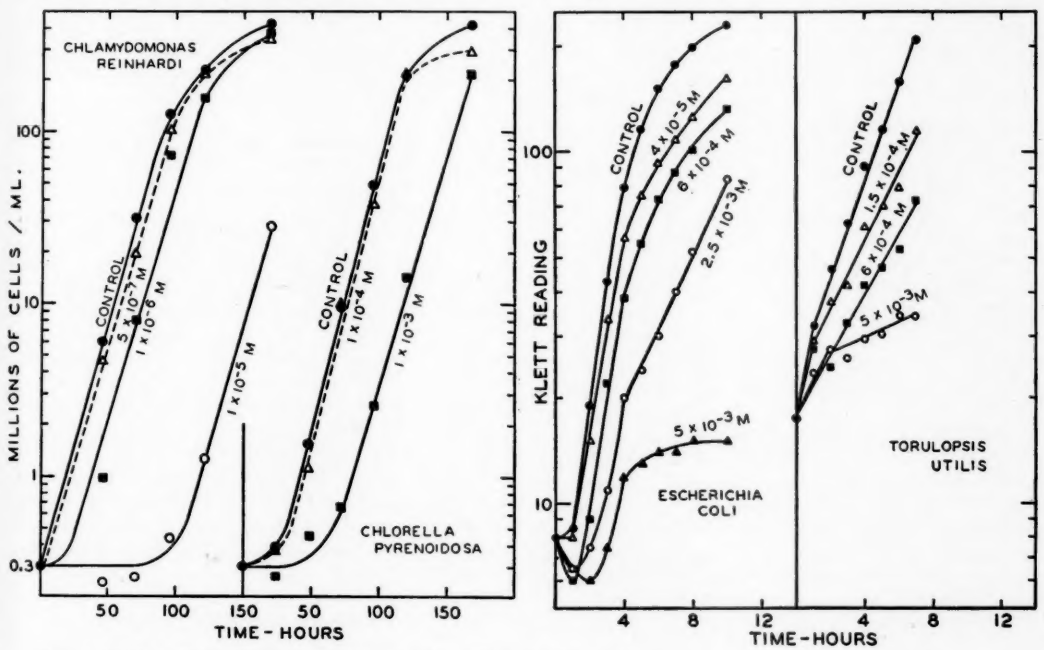



Fig. 5 (*left*). *Chlamydomonas reinhardi* and *Chlorella pyrenoidosa.*
Fig. 6 (*right*). *Escherichia coli* and *Torulopsis utilis.*

not rule out, of course, the existence of mutants at higher concentrations comparable to those frequently used in studies designed to select mutants resistant to a variety of other chemicals. The resumption of growth as a result of a spontaneous decomposition was also checked. The preincubation tests showed no change in the biological activity of the selenomethionine. There remain the two possibilities that the inoculum cells either destroyed the amino acid analogue via an existing detoxification mechanism, or that they were induced to develop a mechanism enabling them to cope with the substance.

The other algal species studied, *Chlorella pyrenoidosa*, first responded to selenomethionine at the relatively high concentration of $1 \times 10^{-8}$ M (fig 5). The exponential rate of division, after a lag of approximately 59 hours, was equal to that of the control; the cultures appeared to be a normal green. The calculated number of mutants, had this growth solely been due to their presence in the inoculum, would be 8,500 in an inoculum of $3.0 \times 10^5$ cells/ml.

The character of growth inhibition in *Escherichia coli* and in *Torulopsis utilis* differed from that observed with the algae. The effect was on the rate of growth rather than on the lag phase (fig 6). At the higher concentrations, cultures of *E. coli* exhibited a decline in optical density for the first hour or two before the onset of exponential growth. The significance of this early dip is unknown. Microscopic examination failed to reveal uncommonly large cells in the yeast cultures, or the filamentous forms of *E. coli* that are observed after treatment with other growth uncoupling agents.

For only two of the microorganisms tested is there some biochemical data on which to base a comparison with *Chlorella vulgaris*. In cultures of a methionine-requiring mutant of *Escherichia coli* the analogue has been shown to substitute entirely for methionine and to support growth and division at a slightly reduced exponential rate through at least 100 generations (10, 11). In *Chlorella pyrenoidosa*, the failure of selenomethionine to exert an effect, at concentrations that act markedly on *Chlorella vulgaris*, can be attributed to another mechanism. Since methionine itself is but negligibly incorporated into the pyrenoidosa proteins and, in contrast to its utilization by *C. vulgaris*, will not serve as a sulfur source for growth (*27*, 28), a parallel situation may hold for selenomethionine.

With respect to the biochemical locus at which selenomethionine inhibits division in *C. vulgaris*, interference in cell wall formation does not appear likely for the following reasons. The occurrence of pectins in algae that lack a middle lamella is uncertain, especially in view of recent analyses which failed to detect uronic acids in the cell wall of *Chlorella pyrenoidosa* (19). Moreover, the analogue, rather than inhibiting transmethylation from methionine, which in higher plants participates in the synthesis of middle lamella components (6, 7, 24, *27*, 28), actually performs as a substrate in rabbit liver and yeast transmethylation enzyme preparations (18). Uncoupling in *Chlorella vulgaris* is more probably caused by a synthesis of altered proteins that lack methionine, in spite of which the cells show striking increases in a number of cellular activities (32). Undoubtedly some of the altered proteins continue to function and lead to the observed growth into giant cells. As precedent for such an interpretation one can cite experiments in which isolated bacterial amylase, containing ethionine, maintained its full activity (37), and particularly the experiments in which a methionine-requiring mutant of *Escherichia coli* used selenomethionine for 100 generations of growth and division (10, 11). Although uncoupled growth in *Chlorella vulgaris* seems to be accomplished by altered enzymes, the fact that division is prevented suggests that the integrity of other proteins, necessary for the division mechanism, must be maintained. Inasmuch as selenomethionine differs from methionine only by the substitution of the sulfur atom, it may be inferred that this integrity requires the methionine sulfur atom.

## Summary

The ability to uncouple growth from division in cells of *Chlorella vulgaris*, a property of selenomethionine, was tested with six other methionine analogues. None were capable of this type of biological specificity. Ethionine and methionine sulfoximine differed in their patterns of growth inhibition but never completely prevented growth. Preincubation of media that contained these analogues markedly reduced their toxicity. Norvaline, methionine sulfoxide, norleucine, and methoxinine caused slight inhibition and only at relatively high concentrations. These last two analogues gave rise to chlorotic cells.

The growth response of four other microorganisms to selenomethionine was also studied, but uncoupling was not detected. *Chlamydomonas reinhardi* and *Chlorella pyrenoidosa* did, however, show increases in lag as the concentration of the selenium analogue was raised. The rate of growth of *Escherichia coli* was slowed, and high concentrations caused a decrease in optical density before the onset of exponential growth. The effect on *Torulopsis utilis* was primarily a decline in the growth rate.

The specific uncoupling action of selenomethionine on cells of *Chlorella vulgaris* is discussed in terms of the known biochemical properties of the analogue. It is proposed that two classes of cellular proteins exist: one involved in growth of the cell and in which methionine may be replaced by selenomethionine; the other more directly concerned with the division mechanism and whose proteins must retain their integrity insofar as replacement of methionine by selenomethionine is concerned.

## Acknowledgments

The author wishes to thank Miss Eva Konrad for her technical assistance, and Mr. Fred Weiter for the experiments with *Escherichia coli*.

## Literature Cited


1. Anderson, E. H. 1946. Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B." Proc. Nat. Acad. Sci., U. S. 32: 120–128.
2. Arnow, P., J. J. Oleson, and J. H. Williams. 1953. The effect of arginine on the nutrition of *Chlorella vulgaris*. Amer. Jour. Bot. 40: 100–104.
3. Block, R. J. and K. W. Weiss. 1956. Amino Acid Handbook. Pp. 252–337. Charles C. Thomas Publ., Springfield, Ill.
4. Brady, L. R. and V. E. Tyler, Jr. 1958. Biosynthesis of hordenine in tissue homogenates of *Panicum miliaceum* L. Plant Physiol. 33: 334–338.
5. Brown, S. A. and R. U. Byerrum. 1952. The origin of the methyl carbon of nicotine formed by *Nicotiana rustica* L. Jour. Amer. Chem. Soc. 74: 1523–1526.
6. Byerrum, R. U. and J. H. Flokstra. 1952. Possible origins of the methoxyl carbon of lignin in barley (*Hordeum vulgare*). Fed. Proc. 11: 193–194.
7. Byerrum, R. U., J. H. Flokstra, L. J. Dewey, and C. D. Ball. 1954. Incorporation of formate and the methyl group of methionine into methoxyl groups of lignin. Jour. Biol. Chem. 210: 633–643.
8. Byerrum, R. U. and R. E. Wing. 1953. The role of choline in some metabolic reactions of *Nicotiana rustica*. Jour. Biol. Chem. 205: 637–642.
9. Cantoni, G. L. 1956. Proceedings of the Third International Congress of Biochemistry, Brussels, 1955. C. Liebecq, ed. Acad. Press Inc., New York. Pp. 233–237.
10. Cohen, G. N. and D. B. Cowie. 1957. Remplacement total de la méthionine par la sélénométhionine dans les protéines d'*Escherichia coli*. Compt. rend. acad. sci., France 244: 680–683.
11. Cowie, D. B. and G. N. Cohen. 1957. Biosynthesis by *Escherichia coli* of active altered proteins containing selenium instead of sulfur. Biochim. Biophys. Acta 26: 252–261.
12. Dubeck, M. and S. Kirkwood. 1952. The origin of the *O*- and *N*-methyl groups of the alkaloid ricinine. Jour. Biol. Chem. 199: 307–312.
13. Green, M. and S. S. Cohen. 1957. Studies on the biosynthesis of bacterial and viral pyrimidines. Jour. Biol. Chem. 225: 387–396.
14. Hase, E., Y. Morimura, and H. Tamiya. 1957. Some data on the growth physiology of Chlorella studied by the technique of synchronous culture. Arch. Biochem. Biophys. 69: 149–165.
15. Hase, E., Y. Morimura, S. Mihara, and H. Tamiya. 1958. The role of sulfur in the cell division of Chlorella. Arch. Mikrobiol. 31: 87–95.
16. Kit, S., C. Beck, O. L. Graham, and A. Gross. 1958. Further studies on the pathways of synthesis of thymine compounds by lymphatic tissues and tumors Jour. Biol. Chem. 233: 944–947.
17. Mehta, R., D. A. Vaughan, S. R. Wagle, K. D. Barbee, S. P. Mistry, and B. C. Johnson. 1959. Role of folic acid and energy intake in nucleic acid synthesis. Jour. Biol. Chem. 234: 625–627.
18. Mudd, S. H. and G. L. Cantoni. 1957. Selenomethionine in enzymatic transmethylations. Nature 180: 1052.
19. Northcote, D. H. and K. J. Goulding. 1958. The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*. Biochem. Jour. 70: 391–397.
20. Olson, B. H. and M. J. Johnson. 1949. Factors producing high yeast yields in synthetic media. Jour. Bacteriol. 57: 235–246.
21. Ordin, L., R. Cleland, and J. Bonner. 1957. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32: 216–220.
22. Parks, L. W. 1958. S-adenosylethionine and ethionine inhibition. Jour. Biol. Chem. 232: 169–176.
23. Remy, C. N. 1959. Metabolism of 2,6-diaminopurine: S-adenosylmethionine as methyl donor for 2-methylamino-6-aminopurine synthesis. Jour. Biol. Chem. 234: 1485–1491.
24. Sager, R. and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardi*. Ann. N.Y. Acad. Sci. 56: 831–838.
25. Sato, C. S., R. U. Byerrum, and C. D. Ball. 1957. The biosynthesis of pectinic acid methyl esters through transmethylation from methionine. Jour. Biol. Chem. 224: 717–723.
26. Sato, C. S., R. U. Byerrum, P. Albersheim, and J. Bonner. 1958. Metabolism of methionine and pectin esterification in a plant tissue. Jour. Biol. Chem. 233: 128–131.
27. Schiff, J. A. 1956. Preliminary studies on the sulfur metabolism of *Chlorella pyrenoidosa* with sulfur-35. Ph.D. thesis, Univ. of Pennsylvania.
28. Schiff, J. A. 1959. Studies on sulfate utilization by *Chlorella pyrenoidosa* using sulfate-$S^{35}$; the occurrence of S-adenosyl methionine. Plant Physiol. 34: 73–80.
29. Schlenk, F. and J. A. Tillotson. 1954. Formation of 5′-ethylthioadenosine from DL-ethionine in yeast. Jour. Biol. Chem. 206: 687–693.
30. Shrift, A. 1954. Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of *Chlorella vulgaris*. Amer. Jour. Bot. 41: 223–230.
31. Shrift, A. 1954. Sulfur-selenium antagonism. II. Antimetabolite action of seleno-methionine on the growth of *Chlorella vulgaris*. Amer. Jour. Bot. 41: 345–352.
32. Shrift, A. 1959. Nitrogen and sulfur changes associated with growth uncoupled from cell division in *Chlorella vulgaris*. Plant Physiol. 34: 505–512.
33. Sribney, M. and S. Kirkwood. 1953. Origin of the methylenedioxy groups of the alkaloid protopine. Nature 171: 931–932.
34. Stern, H. 1956. Sulfhydryl groups and cell division. Science 124: 1292–1293.
35. Stern, H. 1959. Mitosis and metabolic organization. Bot. Rev. 25: 351–384.
36. Tuve, T. W. and H. H. Williams. 1957. Identification of selenomethionine in the proteins of *Escherichia coli* employing the chromatographic "fingerprint" method. Jour. Amer. Chem. Soc. 79: 5830–5831.
37. Yoshida, A. 1958. Studies on the mechanism of protein synthesis: bacterial $\alpha$-amylase containing ethionine. Biochim. Biophys. Acta 29: 213–214.

# IRON NUTRITION OF NICOTIANA TABACUM L. IN RELATION TO RIBOFLAVIN, RIBOFLAVIN-5-PHOSPHATE, AND FLAVIN ADENINE DINUCLEOTIDE CONTENT [1, 2]

GEORGE W. WELKIE AND GENE W. MILLER

UTAH STATE UNIVERSITY, LOGAN

## INTRODUCTION

Under normal conditions of growth, riboflavin is synthesized by all higher plants and is utilized as a prosthetic group by various flavoprotein enzymes (12). Pound and Welkie (11) observed the presence of a yellow pigment in the nutrient solution of tobacco plants grown in the absence of iron. Physical and chemical tests indicated that the pigment was riboflavin, and it was suggested that the deficiency of iron interferes with the utilization of the riboflavin as a prosthetic group of flavoprotein enzymes, which results in the accumulation of riboflavin in the plant and excretion by the roots. Increased synthesis of riboflavin by yeast (*Candida quilliermondia*) grown under conditions of low iron nutrition has been reported by Tanner et al (16) and Burkholder (2).

This research was initiated to investigate further the relation of iron nutrition and flavin concentration in tobacco plants. The appearance and disappearance of riboflavin in nutrient solutions was studied in relation to iron concentration in the nutrient solution and the concentration of riboflavin within whole plants, roots, and leaves. Concentrations of riboflavin, flavin adenine dinucleotide (FAD), and flavinmonophosphate (FMN) in normal and iron-deficient plant parts were determined. Enzyme assays of a non-iron, flavoprotein enzyme, nitrate reductase (10), and a possible iron, flavoprotein enzyme, cytochrome reductase (10), were determined.

## MATERIALS AND METHODS

RIBOFLAVIN, FAD, AND FMN DETERMINATION: The extraction of flavin compounds as outlined by Bessey et al (1) was followed. Tissue from tobacco plants was ground in an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.) with 20 times its volume of ice water and mixed immediately with an equal volume of 20 per cent trichloroacetic acid. After centrifuging, an aliquot of the supernatant was neutralized with 4 M $K_2HPO_4$. A second aliquot of the solution was stored in the dark at 38° C overnight to produce hydrolysis of FAD to FMN. After hydrolysis this sample was neutralized with $K_2HPO_4$. FAD was determined by differences in the fluorescence between the hydrolyzed and unhydrolyzed sample as outlined by Bessey et al (1). Free riboflavin was determined by shaking an aliquot of the unhydrolyzed sample with equal volumes of water-saturated benzyl alcohol. An aliquot of the benzyl alcohol layer was mixed with an equal volume of water, which was 0.05 M with respect to sodium acetate and acetic acid, and 15 volumes of water-saturated toluene. An aliquot of the aqueous layer was then assayed fluorometrically for riboflavin (1). FMN was calculated by subtracting FAD and free riboflavin from total riboflavin (1). The above procedure proved reliable when known concentrations of FMN, FAD, and riboflavin were assayed.

Riboflavin in the nutrient solution was determined fluorometrically (1) and identified by paper chromatography on Whatman No. 3 paper with a solvent of *n*-butanol-water-acetic acid, 5-4-1 (3).

CULTURAL TECHNIQUES: Tobacco seeds were germinated in Vermiculite and the seedlings transferred to a medium of expanded pumice, which was irrigated daily with Hoagland's nutrient solution. After two to three weeks the plants were transferred to polyethylene gallon pots containing Hoagland's nutrient solution minus iron. The normal requirement of iron (4 ppm) was added to some pots which served as controls. All major salts were purified (15) to remove heavy metals and distilled water was run through a mineral deionizer to eliminate iron contamination in the nutrient solution. Solutions were adjusted daily to pH 5.5 with NaOH and HCl. The nutrient cultures were light-free and well aerated.

ENZYME PREPARATION: A crude extract was prepared using the youngest fully-expanded leaves from tobacco. Two grams of leaf tissue was ground with 10 ml 0.05 M Tris (hydroxymethyl aminomethane) buffer at pH 7.4 in a mortar and then homogenized in a Ten Broeck homogenizer. The material was centrifuged at 25,000 × G for 15 minutes and the supernatant used as the enzyme extract. All preparations were carried on at 1 to 3° C. This crude extract was used for enzyme assays in both nitrate reductase and cytochrome reductase studies and contained from 4 to 8 mg protein per ml (7).

ENZYME ASSAYS: Nitrate reductase activity was determined by colorimetric determination (14) of the nitrite formed in 20 minutes at 28° C. The reaction mixture in a final volume of 0.5 ml contained the following constituents in $\mu$ moles (5): 27 pyrophosphate buffer at pH 7.0, 10 $KNO_3$, 0.1 TPNH, 0.04 FMN, 0.5 $NH_2OH$-HCl and extract containing 0.8 to 1.6 mg protein. The reaction was stopped after 20 minutes by the addition of 0.5 ml of 1 per cent sulfanilamide followed by the addition of 0.5 ml of 0.02 per cent *N*-(1-napthyl) ethylenediamine hydrochloride. Two ml of water was added to each tube and the mixtures were centrifuged to remove protein. Optical density was determined at 540 m$\mu$ with a

[1] Received November 18, 1959.

[2] This investigation was supported in part by a National Science Foundation Grant. Approved as Utah Agricultural Experiment Station Paper No. 110.

Beckman DU. Controls were run in each experiment by stopping the reaction at zero time by adding sulfanilamide before the enzyme was added.

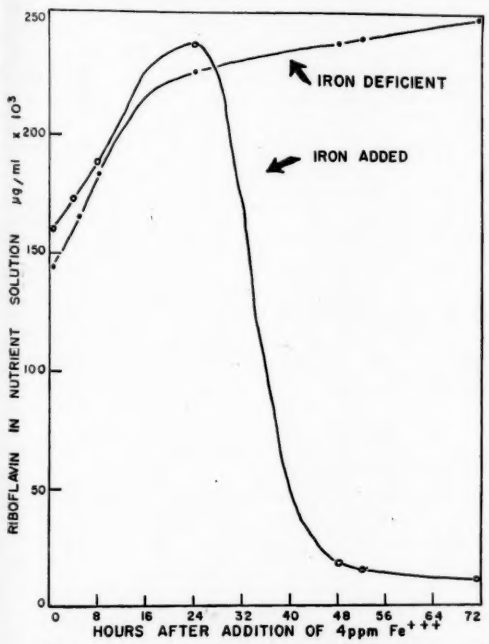
Cytochrome reductase activity was determined as outlined by Mahler et al (10) and Evans (4). The reaction mixture in a final volume of 3 ml contained the following constituents in $\mu$ moles: 50 Tris buffer at pH 7.4, 0.044 oxidized cytochrome c, 0.013 FMN, 0.2 DPNH, and extract containing 0.4 to 0.8 mg protein. The reaction was started with the addition of DPNH and followed spectrophotometrically at 550 m$\mu$ with a Beckman DU. The reaction rate remained linear for at least two minutes.

## Experimental Results

The iron nutrition of tobacco plants influences the amounts of riboflavin detected with time in nutrient solutions (fig 1). The low riboflavin content detected in the control solutions after 4 days plant growth remained unchanged with time, whereas the riboflavin content of the iron-deficient nutrient solutions progressively increased from 4 days until it reached a maximum at 14 days. Fourteen days after initiation of the experiment, the control nutrient solution contained $0.3 \times 10^{-3}$ $\mu$g/ml riboflavin and the iron-deficient nutrient solution contained $249 \times 10^{-3}$ $\mu$g/ml riboflavin.

Adding a full complement of iron to iron-deficient nutrient cultures at a time when chlorosis was perceptible in the tip leaves and riboflavin was rapidly accumulating in the nutrient solution, resulted in a decrease in the riboflavin content in the nutrient solution with continued growth of the plants (fig 2). The presence of iron in the nutrient solution did not result in immediate decrease of the riboflavin. At the time iron was added the riboflavin content was $162 \times 10^{-3}$ $\mu$g/ml. Within 24 hours the nutrient solution contained $237 \times 10^{-3}$ $\mu$g/ml riboflavin after which the level of riboflavin rapidly decreased until it reached the low level of $11 \times 10^{-3}$ $\mu$g/ml 48 hours after adding iron. The riboflavin in the iron-deficient cultures continued to increase during this same period. When plants were removed from iron-deficient nutrient solutions containing riboflavin and iron added, no change in the riboflavin content of the solution occurred. Adding iron (4 ppm) to pure solutions of riboflavin also had no effect on the measurable amount of riboflavin in solution.

The disappearance of riboflavin from nutrient solutions containing iron was studied in relation to normal plants. When concentrations of $220 \times 10^{-3}$ $\mu$g/ml riboflavin were placed in nutrient solutions supporting actively-growing plants, the riboflavin content was reduced to $0.3 \times 10^{-3}$ $\mu$g/ml within 48 hours. If, however, the leaves were removed from plants and the roots allowed to remain in normal nutrient solution containing $275 \times 10^{-3}$ $\mu$g/ml riboflavin



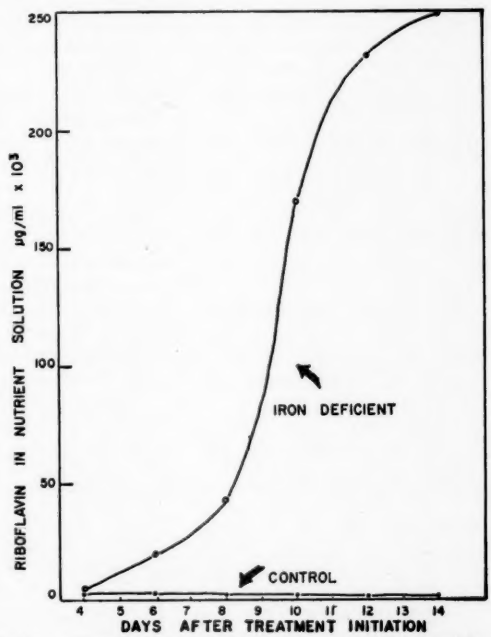




Fig. 1 (*Left*). Accumulation of riboflavin in nutrient solutions supporting the growth of tobacco as influenced by the presence or absence of iron. Control received 4 ppm iron as $FeCl_3$ and the iron-deficient received no iron.

Fig. 2 (*Right*). Change in riboflavin content of iron-deficient nutrient solutions supporting the growth of tobacco as influenced by adding iron ten days after cultures were initiated. Iron was added at the rate of 4.0 ppm as $FeCl_3$, and the control received no iron.

TABLE I

CONCENTRATIONS OF FAD AND RIBOFLAVIN IN TOBACCO LEAVES AS RELATED TO IRON IN NUTRIENT SOLUTIONS

| TREATMENT | DAYS AFTER TREATMENT INITIATION | FAD | RIBOFLAVIN (FMN + FREE RIBOFLAVIN) |
|---|---|---|---|
| | | μ MOLES/G FRESH WT* | |
| Control | 17 | 1.4 | 2.7 |
| Fe deficient | 17 | Trace | 17.1 |
| Control | 21 | 2.7 | 2.3 |
| Fe deficient | 21 | 1.2 | 14.6 |

* Data are averages of three replications.

no decrease in riboflavin content of the nutrient solution was observed in 48 hours. In contrast to this, the placement of excised leaves in normal nutrient solution containing riboflavin resulted in a decrease of the riboflavin content from $530 \times 10^{-3}$ to $22 \times 10^{-3}$ μg/ml within 24 hours.

In order to confirm the hypothesis that iron-deficient tissues were excreting riboflavin as a result of increased concentration of riboflavin in the leaves, samples of normal and iron-deficient leaf tissue were assayed for free riboflavin and FMN, and also for FAD. The FAD concentration of chlorotic leaves was slightly less than the normal, 17, and 21 days after the treatment initiation (table I). However, a six-fold increase of riboflavin (FMN + free riboflavin) was found in the iron-deficient leaves over that found in the control leaves.

Since the flavin content of iron-deficient tissue was markedly increased over that of normal tissue, it was thought desirable to assay flavo-enzymes. The activities of cytochrome reductase and nitrate reductase were determined on normal and iron-deficient tissue. The protein content of chlorotic leaves was 25 to 30 per cent less than that of normal leaves.

Whether the decrease in protein was a general decrease of all protein or a decrease of one specific protein was not known, however, it was presumed that most of the decrease was associated with protein of the chloroplast. Consequently, enzyme activity was based upon dry weight of the tissue. Preliminary experiments showed no significant difference in the water content of normal and chlorotic leaves. Cytochrome reductase activity was 18 to 29 per cent less in extracts from chlorotic leaves than in extracts from normal leaves (table II). Nitrate reductase activity was similar in extracts from both chlorotic and healthy leaves. Had the enzyme activities been calculated on a protein basis, the chlorotic tissue would have yielded greater enzyme activities than the normal tissue.

Since the large increase in total riboflavin in iron-deficient tissue was a composite of both FMN and free riboflavin, further assays were made to determine the content of both FMN and free riboflavin on an individual basis, as well as FAD, in normal and iron-deficient leaf tissue. The tissues were also assayed for nitrate reductase and cytochrome reductase activities. The comparable amounts of FAD, FMN, free riboflavin, and total riboflavin (FAD, FMN, free riboflavin) found in normal and chlorotic leaves at three different periods are shown in table III. There was little difference in the FAD content of chlorotic and normal leaves and the content of FAD showed no correlation with length of treatment. FMN and free riboflavin increased with length of treatment in the chlorotic leaves and concentration of both at all harvest dates was markedly higher in the chlorotic leaves than in the normal leaves. The FMN concentration in chlorotic leaves was greater than that of the free riboflavin at each harvest, the difference being 7 per cent at the first harvest (5 days) 48 per cent at the second harvest (9 days), and 39 per cent at the third harvest (16 days). At 5 days FMN was 43 per cent of the total riboflavin but increased to 55 and 63 per cent of the total riboflavin at 9 and 16 days, respectively, after treatment. Free riboflavin decreased from 40 per cent of the total after 5 days treatment to 30 per cent after 9 days and increased to 38 per cent after 16 days treatment. After 16 days treatment total riboflavin compounds in the chlorotic leaf tissue amounted to more than eight times what was present in the normal leaf tissue. The results with flavo-enzymes were similar to the previous results. Cytochrome reductase activity of the chlorotic leaf extract was reduced 30 to 40 per cent, whereas little difference was noted in the nitrate reductase

TABLE II

CYTOCHROME REDUCTASE AND NITRATE REDUCTASE ACTIVITIES OF EXTRACTS FROM TOBACCO LEAVES AS RELATED TO IRON IN NUTRIENT SOLUTIONS.

| TREATMENT | DAYS AFTER TREATMENT INITIATION | CYTOCHROME REDUCTASE O.D. CHANGE/MIN $\times 10^3$/MG DRY WT TISSUE* | NITRATE REDUCTASE μ MOLES NITRITE FORMED/HR $\times 10^2$/MG DRY WT TISSUE* | |
|---|---|---|---|---|
| | | | COMPLETE | No TPNH |
| Control | 17 | 24 | 10.0 | 0.9 |
| Fe deficient | 17 | 17 | 9.4 | 0.8 |
| Control | 21 | 22 | 7.8 | 1.5 |
| Fe deficient | 21 | 18 | 7.3 | 1.4 |

* Results are averages of three replications.

TABLE III

CONCENTRATION OF FAD, FMN, AND FREE RIBOFLAVIN IN TOBACCO LEAVES AND CYTOCHROME AND NITRATE REDUCTASE ACTIVITIES FROM EXTRACTS OF TOBACCO LEAVES AS RELATED TO IRON IN NUTRIENT SOLUTIONS

| TREATMENT | DAYS AFTER TREATMENT INITIATION | FAD | FMN | FREE RIBOFLAVIN | TOTAL RIBOFLAVIN | CYTOCHROME REDUCTASE | NITRATE REDUCTASE | |
|---|---|---|---|---|---|---|---|---|
| | | μ MOLES PER G FRESH WT* | | | | O.D. CHANGE/MIN × $10^3$/MG DRY WT TISSUE* | μ MOLES NITRITE FORMED/HR × $10^2$/MG DRY WT TISSUE* | |
| | | | | | | | COMPLETE | NO TPNH |
| Control | 5 | 1.2 | 0.1 | 1.4 | 2.7 | | | |
| Fe deficient | 5 | 1.6 | 4.3 | 4.0 | 9.9 | | | |
| Control | 9 | 0.5 | 0.5 | 0.8 | 1.8 | 26 | 5.5 | 1.7 |
| Fe deficient | 9 | 2.8 | 9.6 | 5.0 | 17.4 | 16 | 5.2 | 1.4 |
| Control | 16 | 1.1 | Trace | 1.2 | 2.3 | 34 | 15.4 | 3.7 |
| Fe deficient | 16 | 0.5 | 11.4 | 7.0 | 18.9 | 24 | 14.2 | 3.0 |

* Results are averages of three replications.

activity of comparable chlorotic and normal green leaf tissue.

The concentration of total riboflavin in various tissues of an iron-deficient, chlorotic plant was determined. The concentration of riboflavin was high in all leaves of the iron-deficient plant, which included the extremely chlorotic apical leaves and the non-chlorotic, basal leaves (table IV). The youngest, fully-expanded leaves, which were partially chlorotic, contained a significantly greater amount of riboflavin than any of the other leaves or plant tissues. The stem contained only about one per cent the amount of riboflavin found in the leaves; however, the roots contained about the same as that found in the leaves.

## DISCUSSION

The results clearly indicate that tobacco grown in nutrient solution sufficiently low in iron to induce chlorosis resulted in the excretion of large amounts of riboflavin. It is also of some significance that riboflavin could be detected in small quantities ($0.3 \times 10^{-3}$ μg/ml in nutrient solution containing the normal requirement of iron after tobacco plants were allowed to grow in these solutions for as short a time as 4 days. The concentration of riboflavin in the complete solutions did not, however, increase with time as it did in the iron-deficient solutions. The excretion of riboflavin by the roots of plants may be due to establishment of an equilibrium of the uncomplexed flavin with the external medium. The increased quantities of riboflavin that accumulate in iron-deficient nutrient cultures may be the direct result of higher concentrations of riboflavin within the iron-deficient plants. It is suggested that when riboflavin concentration is increased, larger amounts of riboflavin are translocated to the roots and excreted into the nutrient solution.

TABLE IV

TOTAL RIBOFLAVIN (FAD, FMN, FREE RIBOFLAVIN) IN TISSUE OF CHLOROTIC TOBACCO PLANT GROWING 16 DAYS IN FE-DEFICIENT NUTRIENT SOLUTION

| PLANT TISSUE | CHLOROTIC CONDITION | TOTAL RIBOFLAVIN μ MOLES/G FRESH WT |
|---|---|---|
| 3 Apical leaves | Yellow | 10.6 |
| Next unexpanded leaf | Yellow-green | 10.6 |
| 2 Middle expanded leaves | Yellow-green | 15.4 |
| 2 Basal leaves | Green | 8.0 |
| Stem | ... | 0.1 |
| Roots | ... | 10.4 |

The disappearance of riboflavin from nutrient solutions upon adding iron and continued plant growth can most reasonably be interpreted as reabsorption by the plant. Without plant growth after adding iron, the riboflavin content in the nutrient solution was unchanged. The possible excretion of a reducing compound by growing plants in the presence of iron was considered as an explanation for the disappearance of the riboflavin. This does not seem reasonable, however, in view of the fact that riboflavin is easily oxidized with atmospheric oxygen and the fact that nutrient cultures were sufficiently aerated. Also, the disappearance of riboflavin from cultures containing excised leaves, but not from cultures containing defoliated plants, suggests a movement into the leaves. Adding large amounts of riboflavin to normal nutrient solutions supporting plant growth resulted in the total disappearance of the riboflavin within 48 hours. Assays of the riboflavin content of these plants, however, showed the content was no greater than that of normal control plants that did not receive any riboflavin. It is conceivable that the leaves are the site of degradation of riboflavin, and any excess riboflavin that may be translocated from the roots to the leaves will be degraded and metabolized.

There is no specific report that riboflavin is synthesized in plant leaves but on the basis of its high concentration in leaves and its requirement for excised root growth, the leaves have been considered the site of synthesis (12). Since riboflavin is sub-

ject to photolytic degradation it is possible that much of that which is synthesized within the leaves normally is subject to photolytic degradation. The increased concentration of riboflavin in iron-deficient leaves may be due to reduced degradation, the rate of which is possibly dependent upon the level of iron in the leaves. With respect to this hypothesis, it is of some interest that Sakai (13) reported the acceleration of aerobic photolysis of riboflavin by iron compounds.

Nitrate reductase activity was not appreciably effected by low iron or increases in FMN and free riboflavin. Evans (5) concluded that nitrate reductase from soybean contained FAD as the principal flavin nucleotide. Consequently, one would expect no significant change in nitrate reductase as long as enzyme protein synthesis, FAD, and $NO_3$ levels remained constant in iron-deficient plants.

Cytochrome reductase, an iron-flavoprotein in animal tissue, showed a marked decrease in activity in chlorotic tobacco leaf tissue. In muscle and Neurospora (10) this same iron-flavo-enzyme has been reported to contain FAD as the principal flavin nucleotide. Cytochrome reductase from soybean is equally efficient with FAD or FMN, however, the native flavin nucleotide has not been determined (4). Since FMN is an efficient nucleotide for enzyme activity, one might have expected the large increase of FMN in iron-deficient plants to stimulate enzyme activity. The enzyme activity was, however, significantly reduced. This may have been due partially to the reduced iron level, but it also is probably due to the increased concentrations of FMN and riboflavin which actually inhibited cytochrome reductase activity. Mahler (8) reported that FAD at $10^{-4}$ M and riboflavin at $10^{-3}$ M completely inhibited the enzymatic activity of cytochrome reductase from heart muscle. FMN showed 50 per cent inhibition at $10^{-3}$ M. The levels of FMN and riboflavin in iron-deficient leaves were within the range of concentrations that inhibit cytochrome reductase activity.

It is possible that the increase in total riboflavin in iron-deficient plants contributes to additional metabolic disturbances associated with chlorosis, such as factors influencing growth. Growth substances such as indoleacetic acid and other compounds containing the indole nucleus are rapidly destroyed by a riboflavin-catalyzed photo-oxidation (6). Destruction of these growth substances would severely retard plant growth.

## Summary and Conclusions

Riboflavin apparently is excreted in trace quantities from the roots of tobacco plants grown in nutrient solution, but a deficiency of iron in the nutrient solution results in a 1,000-fold increase in the amount of riboflavin excreted. A deficiency of iron also brings about ten-fold increases in the concentrations of free riboflavin and FMN in tobacco leaves but does not change the FAD level.

The disappearance of riboflavin from the nutrient solution of iron-deficient plants upon adding iron and the continued growth of plants in the nutrient solution indicate that riboflavin is reabsorbed by the plant roots. Adding riboflavin to normal nutrient solution supporting normal plants also results in disappearance of the riboflavin within 48 hours. The inability to note an increase in riboflavin in such plants after 48 hours suggests a possible degradation of the absorbed riboflavin.

The increased free riboflavin and FMN in iron-deficient leaves had no enhancing effect upon the activity of the two flavo-enzymes tested. Cytochrome reductase activity was actually decreased and may be accounted for by an inhibitory effect of riboflavin and FMN.

## Literature Cited

1. Bessey, O. A., O. H. Lowry, and Ruth Love. 1949. The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. Jour. Biol. Chem. 180: 755–769.
2. Burkholder, P. R. 1943. Synthesis of riboflavin by a yeast. Proc. Nat. Acad. Sci., U. S. 29: 166–172.
3. Crammer, J. L. 1948. Paper chromatography of flavin nucleotides. Nature 161: 349–350.
4. Evans, H. J. 1955. Studies on cytochrome reductase in higher plants. Plant Physiol. 30: 437–444.
5. Evans, H. J., and A. Nason. 1953. Pyridine nucleotide nitrate reductase from extracts of higher plants. Plant Physiol. 28: 223–254.
6. Galston, A. W. 1949. Riboflavin-sensitized photooxidation of indoleacetic acid and related compounds. Proc. Nat. Acad. Sci., U. S. 35: 10–16.
7. Lowry, O. H., Nira J. Rosebrough, A. L. Farr, and Rose J. Randall. 1951. Protein measurements with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275.
8. Mahler, H. R. 1955. In: Methods in Enzymology, Vol. II, p. 693. S. P. Colowick and N. O. Kaplan, eds. Academic Press, Inc., N. Y.
9. Mahler, H. R., N. K. Sarkar, L. P. Vernon, and R. A. Alberty. 1952. Studies on nucleotide-cytochrome c reductase. Jour. Biol. Chem. 199: 585–597.
10. Nicholas, D. J. D. 1957. Role of metals in enzymes with special reference to flavoproteins. Nature 179: 800–804.
11. Pound, G. S., and G. W. Welkie. 1958. Iron nutrition of *Nicotiana tabacum* L. in relation to multiplication of tobacco mosaic virus. Virology 5: 371–381.
12. Rosenberg, N. R. 1945. In: Chemistry and Physiology of the Vitamins. Pp. 153–194. Interscience Publishers, Inc., New York.
13. Saki, K. 1956. Influence of metal ions on photolysis of riboflavin. Nagoya Jour. Med. Sci. 18: 222–231.
14. Snell, F. D., and Cornelia T. Snell. 1949. In: Colorimetric Methods of Analysis. D. Van Nostrand Co., New York.
15. Stout, P. R., and D. I. Arnon. 1939. Experimental methods for the study of plants. Amer. Jour. Bot. 26: 144–149.
16. Tanner, F. W., Jr., C. Vojnovich, and F. M. van Laven. 1945. Riboflavin production by Candida species. Science 101: 180–181.

# INFLUENCE OF GIBBERELLIC ACID ON SETTING AND DEVELOPMENT OF FRUITS IN TOMATO[1, 2]

FELIX G. GUSTAFSON

DEPARTMENT OF BOTANY, UNIVERSITY OF MICHIGAN, ANN ARBOR

Considering the total number of investigations concerned with gibberellic acid those dealing with fruits are relatively few (1–13). Gibberellic acid (GA) is capable of inducing parthenocarpy (1, 3, 5, 6, 7, 13), but whether or not fruit setting is increased at the same time is not known (4, 6, 12). Several investigators found no increase in fruit size with GA treatment (4, 6, 10), but investigators did find considerable increase in the size of the grape berry (8, 9, 10, 11). There is thus room for more work with GA on fruiting. In 1957 the writer set up three experiments for studying the effect of GA on setting and development of fruit in *Lycopersicum esculentum* Mill. var. John Baer.

Early in February an experiment with 2 foot John Baer tomato plants in 6 inch pots was set up in the greenhouse. The purpose was to make a comparative study of indoleacetic acid, 2, 3, 6-trichlorobenzoic acid and gibberellic acid on induction of parthenocarpic fruits. These growth regulators were incorporated in lanolin pastes both alone and in combinations with GA in the following concentrations: 1.0 and 0.5 % IAA; 0.1 % 2, 3, 6-TCBA; 1.0 and 0.5 % GA; 0.5 % IAA + 0.5 % GA, and 0.1 % 2, 3, 6–TCBA + 0.25 % GA. These pastes were applied to the cut surface of the pistil of emasculated flower buds, ready to open. Only the first two buds in each cluster were used; the others were removed. Seventy-four mature fruits were harvested. Of these, 16 produced by pollination were seeded; all others were seedless. The seedless fruits produced by applying growth regulators were a little smaller than the seeded ones. The GA treatment alone induced parthenocarpy; adding growth regulators to the GA did not alter the percent setting or the size of the fruits. This is different from the report of Luckwill (3), in which he states that there was a synergistic effect when the growth regulators were added to GA.

In late April a second experiment was started (also in the greenhouse), with 36 plants approximately 30 inches tall. In this experiment there was no attempt to produce parthenocarpic fruits but only to study the effect of GA on setting and size of fruit. Because the plants were not all ready at the same time the experiment was divided into two parts. As soon as the flowers and flower buds were ready, 20 plants were selected; the first three clusters of ten plants were sprayed with 70 ppm GA; ten other plants were sprayed with water.

Several weeks later eight plants were sprayed with 35 ppm GA and eight others with water. At the time of spraying clusters one had a few recently set fruits, flowers, and buds; clusters two had flowers and buds; and clusters three had only small buds. As the fruits matured they were harvested, counted, weighed, and sectioned for observation as to seed and pulp conditions. It is seen in table I that spraying the tomato flowers or buds increased setting but the fruits were smaller and the total yield was decreased by the GA. Many of the GA treated fruits were only partially seeded; some lacked locules.

In late May, 1957, 76 John Baer tomato plants were planted in the field. By June 9 the flowers of the first cluster were opening and at that time this cluster was removed from 46 plants. The flowers and buds of 10 of the remaining 30 plants were sprayed with a solution of 70 ppm GA. A further group of ten was similarly treated with 35 ppm GA,

TABLE I

1ST 3 CLUSTERS SPRAYED WITH 0, 35, OR 70 PPM GIBBERELLIC ACID. ALL 3 CLUSTERS SPRAYED AT SAME TIME

| TREATMENT | NO. PLANTS | TOTAL NO. FRUITS | TOTAL WT FRUITS GRAMS | AVERAGE WT/ FRUIT GRAMS |
|---|---|---|---|---|
| Control | 10 | 55 | 3,850 | 70 |
| 70 ppm GA | 10 | 66 | 2,508 | 38 |
| Control | 8 | 32 | 2,688 | 84 |
| 35 ppm GA | 8 | 45 | 3,375 | 75 |
| | | *Summary* | | |
| Controls | 18 | 87 | 6,525 | 75 |
| GA treated | 18 | 111 | 5,990 | 54 |

Note: At time of spraying the first clusters had a few recently formed fruits, flowers, and buds; clusters two had flowers and buds, and clusters three had only small buds. Many of the third clusters did not set any fruits.

[1] Received November 20, 1959.

[2] Paper from the Department of Botany, University of Michigan, No. 1105.

while the remaining ten were sprayed with distilled water. On June 25 clusters two and three of all 76 plants were sprayed. Plants 1 to 10 and 31 to 40 were sprayed with 70 ppm GA, 11 to 20 and 41 to 57 with 35 ppm GA, and 21 to 30 and 60 to 76 received only water. As the fruits ripened they were harvested and weighed. The number of fruits and their individual weights were carefully recorded per cluster per plant. This was done for five clusters, but only the three that were treated are reported here (table II).

From the table it will be seen that in cluster one GA was effective in increasing the number of fruits produced per cluster and the total weight of fruit per cluster, but not the size of the fruit. The latter fact is not surprising as the size of the fruit in the tomato is associated with the number of fruits in a cluster. The increased setting in cluster one of plants which were sprayed with GA is in accord with the observations of many investigators, including the writer, on plants so treated with growth regulators. The natural setting in the first cluster is always low, but this is materially increased by hormone treatment. The explanation for this may be deficient pollen production and low auxin content due to low temperature early in the season. In clusters two and three GA treatment did not produce an increase in setting or an increase in total weight of fruit produced.

## Discussion

Luckwill (3) found that when flowers of the tomato variety Blaby were sprayed with a solution containing both IAA and GA the size of the resulting fruits was more than twice as large as when sprayed with one of the components alone, that is, there was a synergistic action. This has not been observed with John Baer, although many flowers were treated. The amount of chemical applied to each flower by Luckwill was small and when the concentration of the growth regulators was doubled by the application of both IAA and GA there may have been an excessive growth. Again different varieties of tomato may respond differently.

The present results are in agreement with the findings of other investigators (4, 6,12) of the tomato in that the GA treatment does not increase the size of the fruit. The effect on setting seems to vary, thus two investigators (6, 12) report an increase, whereas one (4) reports no increase. The reports available on seedless grapes (8, 9, 10, 11) all agree that with some concentrations the size of the berry is increased. The photographs of treated clusters show that the clusters are more open, suggesting fewer berries. This in itself would increase the size of the fruit.

This writer is inclined to the view that in the tomato GA is of doubtful value except early in the spring, when natural setting is low. The production of more open clusters in the grape accompanied by larger berries may be of benefit in reducing fungus infection.

## Table II

Summary of Work Done in Field with Gibberellic Acid

| Cluster and treatment | No. plants | Avg. No. fruits/ cluster | Avg. wt/ cluster Grams | Avg. wt/ fruit Grams |
|---|---|---|---|---|
| *Plants with 1st, 2nd, and 3rd clusters sprayed** | | | | |
| 1 | | | | |
| 70 ppm GA | 10 | 3.9 | 435.7 | 111.7 |
| 35 ppm GA | 9 | 2.0 | 226.0 | 113.0 |
| Water | 9 | 1.1 | 133.0 | 119.8 |
| 2 & 3 | | | | |
| 70 ppm GA | 10 | 3.8 | 447.6 | 116.3 |
| 35 ppm GA | 9 | 2.3 | 322.5 | 141.6 |
| Water | 9 | 4.3 | 493.8 | 115.4 |
| *Plants with 1st cluster removed and only clusters 2 and 3 sprayed with gibberellic acid* | | | | |
| 2 & 3 | | | | |
| 70 ppm GA | 10 | 3.2 | 353.7 | 110.5 |
| 35 ppm GA | 18 | 4.3 | 450.0 | 106.1 |
| Water | 16 | 5.6 | 835.0 | 147.7 |

* On June 9 the flowers of the first cluster were sprayed with 70 ppm, 35 ppm GA, or distilled water. On June 25 the flowers in clusters 2 and 3 of these plants as well as those in part 2 of this table were sprayed with 70 ppm, 35 ppm, or distilled water.

## Summary

Gibberellic acid induced the production of seedless fruits on the tomato. When flowers and flower buds of the first three clusters were sprayed with GA, setting was enhanced, but total weight of fruits produced by the plant was reduced. When flowers and flower buds of the first cluster of field grown plants were sprayed with GA the number of fruits set and the total weight of fruit per cluster was increased. This response did not occur in the subsequent clusters similarly treated.

## Literature Cited

1. Jackson, G. A. D. and M. V. Prosser. 1959. The induction of parthenocarpic development in Rosa by auxins and gibberellic acid. Naturwiss. 46: 407–408.
2. Liverman, J. L. and S. P. Johnson. 1957. Control of arrested fruit growth in tomato by gibberellins. Science 125: 1086–1087.
3. Luckwill, L. C. 1959. Fruit growth in relation to internal and external chemical stimuli. In: Cell, Organism, and Milieu, 17th Growth Symposium Pp. 223–251. Ronald Press, New York.
4. Marth, P. C., W. V. Audia, and J. W. Mitchell. 1956. Effect of gibberellic acid on growth and development of plants of various genera and species. Bot. Gaz. 118: 106–111.
5. Persson, A. and L. Rappaport. 1958. Gibberellin-induced systemic fruit-set in a male sterile tomato. Science 127: 816.
6. Rappaport, L. 1957. Effect of gibberellin on growth, flowering, and fruiting of the Earlypack

tomato, *Lycopersicum esculentum*. Plant Physiol. 32: 440–444.
7. SACHAR, R. C. and M. KAPVOR. 1959. Gibberellin in the induction of parthenocarpy in Zephyranthes. Plant Physiol. 34: 168–170.
8. STEWART, W. S., D. HALSEY, and F. T. CHING. 1958. Effect of potassium salt of gibberellic acid on fruit growth of Thompson seedless grape. Proc. Amer. Soc. Hort. Sci. 72: 165–169.
9. WEAVER, R. J. 1958. Effect of gibberellic acid on fruit-set and berry enlargement in seedless grapes of *Vitis vinifera*. Nature 181: 851–852.
10. WEAVER, R. J. and S. B. MCCUNE. 1959. Response of certain varieties of *Vitis vinifera* to gibberellin. Hilgardia 28: 297–350.
11. WEAVER, R. J. and S. B. MCCUNE. 1959. Effect of gibberellin on seedless *Vitis vinifera*. Hilgardia 29: 247–275.
12. WITTWER, S. H. and H. J. BUKOVAC. 1957. Gibberellins and higher plants: X. Field observations with certain vegetable crops. Mich. State Univ., Agr. Exp. Sta., Quarterly Bulletin 40: 352–364.
13. WITTWER, S. H., M. J. BUKOVAC, H. M. SELL, and L. E. WELLER. 1957. Some effect of gibberellin on flower and fruit setting. Plant Physiol. 32: 39–41.

# INFLUENCE OF BORON ON ENZYMATIC REACTIONS ASSOCIATED WITH BIOSYNTHESIS OF SUCROSE[1, 2]

W. M. DUGGER, JR[3], AND T. E. HUMPHREYS

It has been proposed that boron enhances the uptake and transport of sugars in plants (9, 10, 24). The hypothesis that this effect was caused by the formation of a borate-sugar complex which facilitates the absorption and transport of added sugar or photosynthate, however, has not been accepted by all investigators (17, 22).

In addition to the proposed effect of boron on uptake of sugar in plants it has been suggested that the element may influence transport of sugars by inhibiting starch phosphorylase (7). This observation has prompted a more comprehensive investigation of the carbohydrate metabolism of plants as influenced by boron. The phase of this work reported here is concerned with the enzymatic reactions of UDPG and sucrose biosynthesis as recently developed by Leloir and coworkers (4, 14, 20) and others (1, 8, 11, 21, 26, 27, 28), and the effect of boron on these reactions. These reactions are:

I. Uridine triphosphate (UTP) + Glucose-1-phosphate (G-1-P) $\rightleftharpoons$ Uridine diphosphate glucose (UDPG) + pyrophosphate (PP)

II. A. UDPG + fructose $\rightleftharpoons$ Sucrose + Uridine diphosphate (UDP)

B. UDPG + Fructose-6-phosphate (F-6-P) $\rightleftharpoons$ Sucrose-phosphate + UDP

III. UDP + Adenosine triphosphate (ATP) $\rightleftharpoons$ UTP + Adenosine diphosphate (ADP)

## MATERIALS AND METHODS

Seven day-old pea seedlings (*Pisum sativum* L., var. Emerald), grown in the dark at 23° C, were harvested and immediately frozen in liquid air. While frozen the seedlings were ground to a powder (hereafter referred to as homogenate), quickly separated into 1 gram samples, and transferred to reaction flasks containing 0.5 ml of 2 M Tris (hyroxymethyl)-aminomethane buffer pH 7.2, 0.4 ml of 0.1 M G-1-P, 0.2 ml of 0.1 M $MgCl_2$, 0.4 ml of 0.02 M UTP, 0.2 ml of 0.1 M ATP, 0.4 ml of 0.1 M fructose-$C^{14}$ (specific activity of 1 μc/mg), 0.1 ml of yeast inorganic pyrophosphatase, and sufficient water or 0.1 M boric acid to make a total volume of 4.2 ml. In the center well of the flask was placed 0.2 ml 20 % KOH. Immediately after adding the ground plant material, the flask was stoppered and placed in a water bath adjusted to 35° C. After an incubation period of 4 hours, 20 ml of boiling 90 % ethanol was added to stop the reaction and the mixture was allowed to simmer on the hotplate for 5 minutes. The suspension was filtered and the residue washed with hot 83 % ethanol. The filtrate was diluted to 50 ml before 10 ml aliquots were removed and concentrated to 3 ml. After clearing the filtrate with the $Ba(OH)_2$ and $ZnSO_4$ method of Nelson (18), it was assayed for sucrose using chemical and chromatographic techniques. The chemical procedures were; A. Resorcinal method of Roe (23) as modified by Cardini, et al (4) to measure sucrose and, B. Specific glucose oxidase method (7, 29) to measure glucose before and after inverting the sucrose. Chromatograms were developed in *n*-propanol-water-$NH_4OH$ (16:3:1) (27) and the

[1] Received November 25, 1959.
[2] Florida Agricultural Experiment Station Journal Series, No. 782. This work was supported, in part, by the United States Atomic Energy Commission.
[3] Present address: Air Research Laboratory, Citrus Experiment Station, Riverside, Cal.

sugars were located by spraying with *p*-anisidine. The areas on the chromatograms containing the sugars were cut into 2 cm strips and the sugars eluted with water. The sugars were assayed chemically as just described and the products of hydrolysis were chromatographed on paper and the $R_f$'s obtained compared to known glucose and fructose. Radioactivity was determined in a windowless flow counter. Other chromatograms were placed in contact with Eastman no-screen X-ray film for radioautographic detection. In experiments where glucose-$C^{14}$ was used instead of fructose-$C^{14}$, hexokinase and phosphoglucomutase were also added to the reaction mixture.

Uncleared aliquots of the alcohol solution were concentrated and chromatogramed in ethanol-molar-ammonium acetate (7:3) pH 7.5 (20) to separate nucleotides. These compounds were detected by UV light. Areas showing fluorescence were cut out, eluted, and the eluate assayed for radioactivity. UDPG content was also determined enzymatically with UDPG dehydrogenase.

Pea seeds were ground and extracted with acetone and ether as described by Turner (27). The dried powder was extracted with 0.2 M $NaHCO_3$ for 3 hours at room temperature, and the larger suspended particles were removed by low speed centrifugation. The supernatant was centrifuged at 20,000 G and the precipitate discarded. Cold, neutralized, saturated $(NH_4)_2SO_4$ was added to the supernatant until the mixture was 80 % saturated and the precipitate collected by centrifugation (20 min at 20,000 G). The precipitate was dissolved in 10 ml of water and dialyzed against 0.025 M phosphate buffer, pH 7, for 24 hours. This preparation will be referred to as A.S. preparation. Contrary to the results of Turner (27) activity of the enzymes was not lost by dialyzing longer than 6 hours. This preparation from pea seeds contained UDPG pyrophosphorylase, UDPG transglycosylase (saccharese), UDP kinase, myokinase, and nucleoside monophosphate kinase.

Young sugar cane seedlings, (*Saccharose officinarium* var. CP50–28), grown in soil under greenhouse conditions, were harvested 3 to 4 weeks after the stem bud germinated. The above ground parts were removed from the seed piece, cut into small sections, and immediately frozen in liquid air. The frozen material was ground with a large mortar and pestle and transferred to the reaction flasks for incubation with labeled sugars plus the same addenda that was used with the pea plant homogenate.

When A. S. preparations were to be made, the frozen material was transferred to a square of nylon cloth placed in a funnel. As the material thawed, water was added (1 ml/gm tissue) prior to expressing the fluid from the tissue. The filtrate was centrifuged at 20,000 G and solid $(NH_4)_2SO_4$ slowly added with stirring to 80 % saturation. The pH was maintained at 7 to 7.2 by adding 14 % $NH_4OH$. The precipitate was separated by centrifugation and dissolved in water. After dialyzing against 0.025 M phosphate buffer, pH 7, the preparation was stored in a deep freeze. UDPG transglycosylase activity, but not UDPG pyrophosphorylase activity was destroyed by freezing and thawing repeatedly.

The reaction mixture used to determine UDPG pyrophosphorylase activity consisted of 0.1 ml of 2 M Tris buffer, pH 7.2, 0.1 ml of 0.1 M G-1-P, 4.5 μmoles of UTP, 0.05 ml of inorganic pyrophosphatase, and 0.05 ml of 0.1 M $MgCl_2$. One-tenth ml of A. S. preparation (0.8–1.6 μg N) was added to start the reaction after the reaction mixture was diluted to 0.9 ml with water or 0.1 M borate, pH 7.2.

UDPG transglycosylase was assayed by adding 0.1 ml of the A. S. preparation (0.2–0.5 mg N) to 0.1 ml of 2 M Tris buffer pH 7.2, 0.1 ml of 0.1 M fructose or F-6-P, 4.5 μmole UDPG, 0.05 ml of 0.1 M $MgCl_2$ and sufficient water or borate solution to make a total volume of 1.0 ml.

UDP kinase activity was determined by adding the A. S. preparation to the reaction mixture containing 0.1 ml of 2 M Tris buffer, pH 7.2, 1 μmole UDP, 0.05 ml of 0.1 M $MgCl_2$, 0.05 ml of 0.1 M ATP, and sufficient water or borate solution to make a total volume of 1.0 ml. Where this reaction was coupled to the UDPG pyrophosphorylase reaction and assayed by the UDPG dehydrogenase method, 0.1 ml of 0.1 M G-1-P was added. If the method of Berg and Joklik (2) was used, G-1-P was not added.

In the coupled reaction of sucrose synthesis from UTP, G-1-P and fructose, these materials were added at the same levels as in the previous assay methods. $MgCl_2$, ATP, and A. S. preparation were also added at the same level as in the kinase studies.

UDP, UDPG, UMP, UTP, Zwischenferment, hexokinase, and glucose-1-phosphate were supplied by Sigma Chemical Co.; ATP was obtained from Schwarz Laboratories. UDPG dehydrogenase was initially purchased from Sigma Chemical Co., but a more purified preparation was later made from fresh calf liver (25). Inorganic pyrophosphatase was prepared from bakers yeast by a modification of the method used by Heppel and Hilmoe (13). Adenylate kinase, 5′-adenylic acid deaminase, and phosphoglucomutase were prepared from rabbit muscle (5, 16, 19).

UDPG pyrophosphorylase assays were conducted in two ways; the first involved reacting UDPG and PP with the A. S. preparation. The G-1-P formed by the reaction could be measured by coupling with the phosphoglucomutase, glucose-6-phosphate dehydrogenase reactions and observing the change in $OD_{340}$ as TPN was reduced (15). The second method involved the oxidation of the produced UDPG to UDP-glucuronic acid by the enzyme UDPG dehydrogenase when the A. S. preparation was incubated with G-1-P and UTP. The change in optical density as DPN was reduced was followed at 340 mμ (25).

UDPG transglycosylase was assayed by measuring the formation of sucrose when UDPG and fructose or fructose-6-phosphate were incubated with the A. S. preparation. UTP and G-1-P were sometimes used as sources of UDPG if the UDPG pyrophosphorylase reaction was coupled to the UDPG transglycosylase

reaction. Sucrose was determined by the method of Roe (23) after fructose was destroyed by boiling 10 minutes in 0.2 *N* NaOH (4). In later experiments sucrose was hydrolyzed with invertase at pH 4.9 and the glucose determined by the specific glucose oxidase method previously reported (7). This method took longer than the resorcinol method but did avoid the disadvantage of color formations from the reactants. Blanks with boiled A. S. preparation or without UDPG gave readings of 0 to 10 on the Klett colorimeter after 24 hours incubation as compared to readings of 50 to 70 for the reagent blanks by the method of Roe. In the results given in the tables of this report sucrose was estimated by this latter method.

UDP kinase was assayed by reacting UDP and ATP and measuring the formation of UDPG in the presence of excess glucose-1-phosphate and inorganic pyrophosphatase. The method of Berg and Joklik (2) was also used to show the presence of UDP kinase. This involved the reactions:

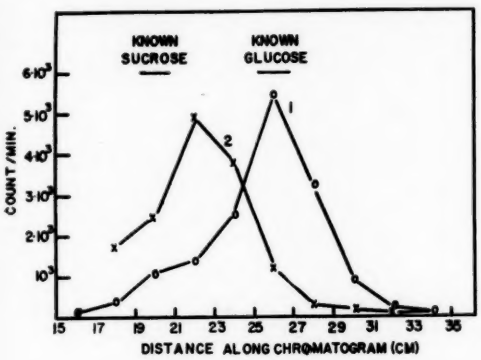
$$UDP + ATP \rightleftharpoons UTP + ADP \text{ (UDP kinase)}$$

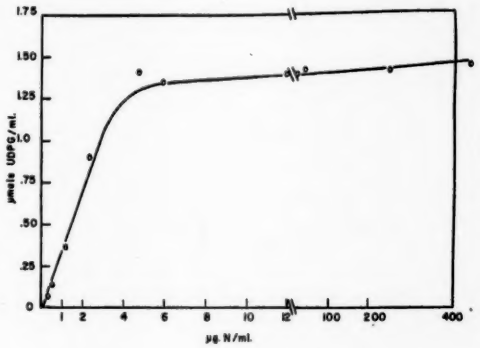
$$2\ ADP \rightleftharpoons ATP + AMP \text{ (adenylate kinase)}$$

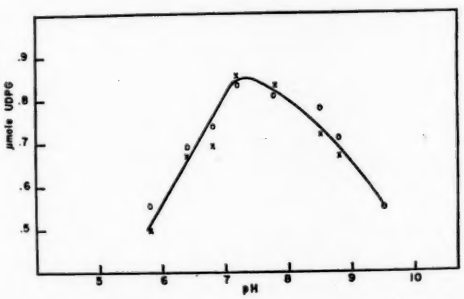
$$AMP \rightleftharpoons IMP \text{ (inosine monophosphate)} + NH_3 \text{ (adenylic acid deaminase)}$$

The reaction was followed by measuring the decrease in absorption at 265 mμ caused by the deamination of AMP.

Phosphorus was determined by the method of Dryer, et al (6), and nitrogen by Nesslerization.



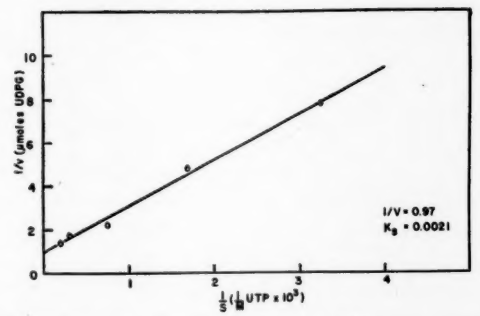




FIG. 1. Radioactive assay of 2 cm strips cut from chromatograms of a reaction mixture in which sugar cane seedling homogenates were incubated with $C^{14}$ glucose and other constituents. Known glucose and sucrose areas on the chromatograms are given for reference. 1. Reaction mixture contained no boron; 2. Reaction mixtures contained 10 μmoles boron/ml.

FIG. 2. Relationship of protein nitrogen of the pea enzyme preparation on the synthesis of UDPG by UDPG pyrophosphorylase. UTP concentration, 4.5 μmole/ml.

FIG. 3. Effect of pH of the reaction mixture on the synthesis of UDPG by UDPG pyrophosphorylase from pea seed preparation. Tris buffer adjusted to indicated pH by HCl.

FIG. 4. Effect of UTP concentration on the rate of UDPG synthesis. The reaction mixtures were as described under Materials and Methods.

## Results and Discussion

The incorporation of glucose-$C^{14}$ into sucrose by sugar cane seedling homogenate was qualitatively indicated by chromatographic and radioautographic methods. The radioactivity of sucrose eluted from chromatograms indicated quantitatively the influence of boron on sucrose synthesis. Boron more than doubled the synthesis of sucrose from glucose-$C^{14}$, UTP, and fructose (fig 1). Hexokinase and phosphoglucomutase in the reaction mixture enhanced the formation of sucrose from glucose-$C^{14}$. Previous studies have shown that the activity of hexokinase from yeast or pea seedlings is unaffected by levels of borate used in these experiments (7), nor does boron affect the enzymatic activity of rabbit phosphoglucomutase that was added to these systems to facilitate the synthesis of $C^{14}$ glucose-1-phosphate. In other experiments the spots corresponding to the 19 to 21 cm portion of the chromatograms (fig 1) were eluted and sucrose identified colorimetrically and chromatographically as described in Materials and Methods. Table I shows the results of another experiment in which the influence of specific co-factors on the synthesis of sucrose was studied. The phosphorylation and transformation of glucose to glucose-1-phosphate by added enzymes and ATP resulted in increased labeling of sucrose by the sugar cane material. Borate also increased the synthesis of sucrose in this experiment as shown in the last row of the table.

Fructose-$C^{14}$ was incorporated into sucrose by ground sugar cane and pea seedling homogenates as well as glucose-$C^{14}$. As glucose-1-phosphate was included in the reaction mixture, it was unnecessary to add hexokinase and phosphoglucomutase to obtain maximum labeling of sucrose from labeled fructose. Table II gives the results of an experiment in which ground pea seedling homogenate was incubated with fructose-$C^{14}$ and other necessary ingredients for sucrose synthesis. Chemical and radioactive assay of the sucrose formed in 4 hours indicated that borate increased the synthesis. The decrease in sucrose concentration from the initial value may possibly be accounted for by the respiration of the plant material during the 4 hours of incubation at 35° C, or by invertase action. However, it was found that boron had no effect upon the activity of commercial preparations of invertase. This was thoroughly checked prior to using the invertase and glucose oxidase assay method for determining sucrose synthesis.

It was also found that boron had no effect on hexokinase or on phosphoglucomutase. These enzymes along with invertase would be implicated in the initial reactions leading to sucrose degradation. Thus, it appears that boron affects the synthesis of sucrose. The appearance of $C^{14}$ in sucrose is no indication of net synthesis as sucrose utilizing and sucrose synthetic reactions are occurring. The data of table II show that there was no correlation between total sucrose and $C^{14}$ content of the sucrose. The chemical methods of sucrose estimation are more reliable than the chromatographic method.

Results with the homogenates pointed to a possible relationship between boron and enzyme systems associated with sucrose synthesis. As mentioned in the previous section, A. S. preparations from sugar cane seedlings and pea seeds contained both UDPG pyrophosphorylase and UDPG transglycosylase. The A. S. preparation from sugar cane was less active than that from pea seeds, and on storage at −16° C the activity decreased at a greater rate than with the pea seed A. S. preparation. Therefore, the results reported in this phase of the work will be from experi-

Table I

Effect of Cofactors on Synthesis of Sucrose by Ground Sugarcane Leaves

| Experiment | Relative radioactivity | |
|---|---|---|
| | Sucrose* % | UDPG** % |
| 1. Complete | 100 | 0 |
| -UTP | 11 | 0 |
| -fructose | 11 | 61 |
| -hexokinase and phosphoglucomutase | 5 | 0 |
| 2. Complete | 100 | |
| -ATP | 46 | |
| -UTP | 57 | |
| -fructose | 56 | |
| 10 μmole B/ml | 176 | |

* Chromatographed in propanol-water-$NH_4OH$ (16:3:1)

** Chromatographed in ethanol-ammonium acetate (7:3) pH 7.5

Sucrose and UDPG spots eluted from chromatograms prior to radioactive assay.

Complete reaction mixture. See Materials and Methods.

Table II

Influence of Boron on Synthesis of Sucrose-$C^{14}$ from Fructose-$C^{14}$ by Pea Seedlings Homogenate

| Treatment | Sucrose (μmole/gm) | Sucrose chromatographed (c/m × $10^3$)* |
|---|---|---|
| Pea seedlings (before incubation) | 8.5 ± 0 | |
| Homogenate without boron (after incubation) | 5.5 ± 0.5 | 6.6 |
| 5 μmole B/ml (after incubation) | 9.5 ± 0.5 | 15.2 |
| 10 μmole B/ml (after incubation) | 10.5 ± 1.2 | 11.0 |
| 15 μmole B/ml (after incubation) | 10.0 ± 0.3 | 15.8 |

* Chromatographed in propanol-water-$NH_4OH$ (16:3:1). Sucrose area eluted and counted in windowless flow counter.

TABLE III

INFLUENCE OF BORON ON SYNTHESIS OF SUCROSE BY ENZYME PREPARATIONS FROM PEA SEEDS*

| BORON CONC (μMOLE/ML OF DIGEST)** | ENZYME PREPARATION | |
|---|---|---|
| | 1 | 2 |
| | SUCROSE (μMOLE/MGN) | |
| 0 | 0.60 | 0.72 |
| 5 | 0.45 | 0.69 |
| 10 | 0.52 | 0.56 |
| 20 | 0.32 | 0.52 |

* Incubation time, 30 minutes, temperature, 35° C.
** Reaction mixture. See Materials and Methods section.

ments using A. S. preparation from pea seeds. These results will also be limited to those experiments in which fructose was included as the acceptor of the glucose from UDPG in the transglycosylase reaction due to the observation that sucrose-phosphate synthesis from both pea seed and sugar cane seedling A. S. preparation was much less active than sucrose synthesis. This is in agreement with the results of Bean and Hassid (1) and Leloir and Cardini (14). Only after repeated extraction with 0.05 M ammonium sulfate were these latter investigators able to obtain an enzyme from wheat germ more active with fructose-6-phosphate as the acceptor of glucose than with fructose. The additional chemical steps necessary to estimate sucrose-phosphate synthesis in these experiments introduced errors often as large as the quantity of sucrose-phosphate determined.

With boron included in the reaction mixture, sucrose synthesis was inhibited (table III). In contrast to the results obtained using homogenates, boron caused a decrease in the amount of sucrose synthesized by the A. S. preparation. If the digests were incubated for 24 hours under a layer of toluene, or if antibiotics were included to prevent microorganism growth, there was little if any effect of boron on sucrose synthesis.

Contrary to the findings by Turner (27) for a similar preparation from pea seeds, it was found that DPN was not necessary for maximum sucrose synthesis, and further that ATP enhanced the synthesis in the coupled reaction (UTP + G-1-P + fructose $\rightleftharpoons$ UDP + PP + sucrose). In the experiments reported by Turner (27), 0.2 mg DPN and 34 μmoles ATP were included per 0.9 ml digest. The enzyme preparation was dialyzed for 2 hours in 25 mM phosphate buffer (pH 8) and the digest time was 19 hours. In experiments reported in this paper, where ATP inhibited the individual reactions and enhanced the coupled reaction, 0.25 mg DPN and 10 μmoles ATP were included per ml of digest. The A. S. preparation was dialyzed 24 hours in 25 mM phosphate buffer and the digest time was for shorter periods. In reaction periods longer than 12 hours it was found necessary to add a layer of toluene to the reaction mixture or include antibiotics to prevent the metabolic utilization of sucrose by microorganisms. Nondialyzed or 2,4, and 6 hour dialyzed A. S. preparations from pea seeds reduced DPN; this was enhanced by adding cysteine. In a later paper by Hatch and Turner (12) it was shown that the 80 % ammonium sulfate precipitate from pea seeds contained all the enzymes necessary for oxidation of hexose to $CO_2$ and ethanol. The enhanced production of $CO_2$ by added DPN is associated with glycolysis and, in some way, as reported by Hatch and Turner (12), influenced sucrose synthesis.

Because of the inconsistent results between the experiments using homogenates and those using A. S. preparations, a more thorough investigation of the individual reactions concerned with sucrose synthesis was attempted.

The A. S. preparation catalyzed the reversible reaction UTP + G-1-P $\rightleftharpoons$ UDPG + PP (15). At the concentration used in the overall reaction where sucrose synthesis from UTP, G-1-P and fructose was measured, 0.27 to 0.47 mg protein N was added with each 0.1 ml of enzyme preparation. However, in order to investigate the UDPG pyrophosphorylase reaction a 1 : 300 dilution of the enzyme was necessary. Figure 2 shows the rate of UDPG formation as a function of A. S. preparation concentration at the level of reactants used in the assay.

The optimum pH for this reaction was 7.2 to 7.4. However, there was an appreciable rate of reaction at lower and higher pH values (fig 3). Other in-

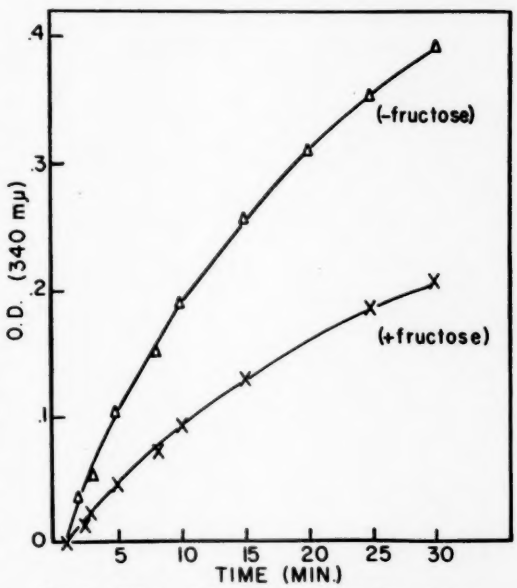



FIG. 5. Effect of fructose in the reaction mixture on the apparent UDPG synthesis. The change in $OD_{340}$ as DPN is reduced by UDPG dehydrogenase is proportional to the concentration of UDPG.

TABLE IV

INFLUENCE OF BORON ON BIOSYNTHESIS OF UDPG BY EXTRACT FROM PEA SEEDS

| BORON CONC (μMOLE/DIGEST)* | UDPG CONC μMOLE/10 μG N) | |
|---|---|---|
| | 1 | 2 |
| 0 | 2.6 | 3.0 |
| 2 | 2.5 | 4.3 |
| 5 | 3.9 | 4.9 |
| 10 | 5.1 | 5.9 |
| 20 | 5.1 | 6.0 |
| 40 | | 5.5 |

* Reaction mixture. See Materials and Methods section.

vestigators have reported a similar pH optimum for this enzyme from plants (8,15). Only by diluting the enzyme 1:300 was it possible to use the UDPG dehydrogenase method of assay (25) without noticeable pyrophosphorylase activity during the 15 to 20 minutes it took to assay for UDPG.

The rate constants for enzyme activity, as dependent upon UTP concentration are given in figure 4. The value of 0.0021 for $K_s$ is approximately ten times the value Ginsburg (11) reported for the reverse reaction catalyzed by a purified enzyme from Mung bean. When fructose was included in the reaction mixture the apparent synthesis of UDPG was less (fig 5). This decrease was caused by the utilization of UDPG in sucrose synthesis. In short time experiments and without ATP in the reaction, this decrease in UDPG when fructose was added to the reaction mixture approximated the quantity of sucrose synthesized by UDPG transglycosylase.

UDPG synthesis in the presence of boron was enhanced. Table IV shows two experiments where approximately 100 per cent increase in UDPG formation in 1 hour was obtained by adding 10 and 20 μmole B/ml.

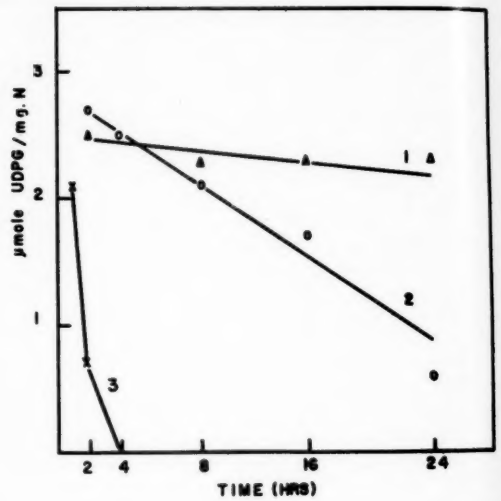
If the UDPG concentration was measured against time in the original reaction mixture where sucrose was synthesized from UTP, G-1-P and fructose in the presence of ATP the influence of boron appears to be that of maintaining UDPG in the mixture (see fig 6). If ATP was not included the UDPG value rose rapidly to a maximum and decreased to zero within 4 hours. This indicates that the A. S. preparation contains UDP kinase and that UDPG is resynthesized from UDP and ATP via UTP. An investigation as to what effect ATP, DPN, and $Mg^{++}$ alone and in combination might have on UDPG synthesis shows

TABLE V

INFLUENCE OF CO-FACTOR ADDITION ON UDPG SYNTHESIS BY UDPG PYROPHOSPHORYLASE FROM PEA SEEDS*

| REACTION MIXTURE** | UDPG (μMOLE/10 MG N) |
|---|---|
| Complete | 2.3 |
| +DPN, $-Mg^{++}$ | 0.3 |
| +ATP, $-Mg^{++}$ | 0.3 |
| +DPN, $+Mg^{++}$ | 1.6 |
| +ATP, $+Mg^{++}$ | 1.3 |
| $+Mg^{++}$, +ATP, +DPN | 1.5 |

* Incubation was for one hour at 35° C.
** Reaction mixture. See Materials and Methods section.



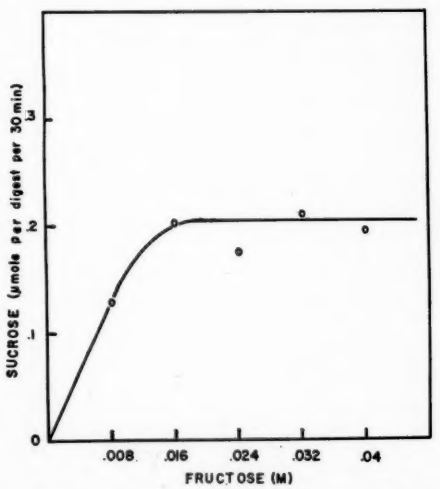




FIG. 6. Effect of ATP and boron on the level of UDPG maintained in the reaction mixture over a 24 hour reaction period. 1. ATP and boron, 10 μmole/ml; 2. ATP; 3. No ATP. The reaction mixtures were as described under Materials and Methods.

FIG. 7. Effect of fructose on the synthesis of sucrose by pea enzyme preparation. The reaction mixtures were as described under Materials and Methods.

TABLE VI

INFLUENCE OF BORON ON SYNTHESIS OF SUCROSE FROM UDPG AND FRUCTOSE BY AN ENZYME PREPARATION FROM PEA SEEDS

| REACTION MIXTURE* | BORON CONC (μMOLE/ML) | SUCROSE (μMOLE/MG N) |
|---|---|---|
| —fructose | 0 | 0.15 ± 0.2** |
| complete | 0 | 1.3 ± 0.1 |
| " | 5 | 1.2 ± 0.1 |
| " | 10 | 1.0 ± 0.0 |
| " | 20 | 0.85 ± 0.05 |

* Reaction mixture. See Materials and Methods section.
**Average of duplicate experiments.

that ATP inhibits the UDPG pyrophosphorylase reaction (see table V). When this reaction is coupled to the transglycosylase and kinase reactions, however, ATP is necessary to maintain UDPG concentration (fig 6) and results in greater sucrose synthesis.

UDPG transglycosylase activity was much less per unit of protein nitrogen than pyrophosphorylase activity. In all experiments where the two reactions were coupled together, UDPG was rapidly synthesized whereas sucrose was synthesized more slowly.

The pH optimum for transglycosylase activity is 7.2 as was also shown for preparations from wheat germ by Cardini et al (4). The effect of fructose concentration on the synthesis of sucrose is shown in figure 7. Boron inhibits this reaction (table VI). The magnitude of inhibition, however, is less than the magnitude of stimulation in UDPG synthesis. Table VII shows the effect of ATP and $Mg^{++}$ concentration on the synthesis of sucrose by UDPG transglycosylase. As shown in this table and table V, ATP inhibits synthesis of UDPG and sucrose in the single reactions, but in the coupled system the addition of ATP facilitates the resynthesis of UDPG and the synthesis of sucrose. When the UTP concentration was reduced to a value less than substrate amounts (0.85 μmole/ml digest) the influence of ATP was more apparent (see table VIII) on UDPG resynthesis and sucrose synthesis. With ATP, the level of UDPG was maintained for 4 hours, whereas with no ATP included in the digest the UDPG was utilized in the transglycosylase reaction and not resynthesized. Sucrose synthesis was approximately doubled when ATP was included in the reaction.

TABLE VII

INFLUENCE OF ATP AND $Mg^{++}$ ON SYNTHESIS OF SUCROSE FROM UDPG AND FRUCTOSE BY AN ENZYME PREPARATION FROM PEA SEEDS

| CONCENTRATION* (μMOLE/ML OF DIGEST) | ATP | $Mg^{++}$ |
|---|---|---|
| | SUCROSE | (μMOLE/ML) |
| 0 | 5.7 | 3.8 |
| 2 | 5.9 | 5.5 |
| 4 | 4.2 | 6.1 |
| 6 | 4.0 | 6.1 |
| 8 | 4.4 | 6.3 |
| 10 | 2.3 | 4.2 |
| 20 | 2.3 | 4.6 |

* Reaction mixture. See Materials and Methods section. When ATP was varied, 5 μmoles of $Mg^{++}$ was added. When $Mg^{++}$ was varied, ATP was omitted.

An attempt was made to characterize UDP kinase, the action of which was implicated in the experiments reported above. The synthesis of UDPG was doubled when 10 and 20 μmoles of borate were included in the reaction. This does not conclusively show an effect of boron on the kinase since UDPG synthesis from UTP and G-1-P was increased by boron. The formation of ADP from ATP and UDP was increased when boron was included in the initial kinase reaction mixture. The kinase was assayed by measuring UDPG formed in the presence of G-1-P, UDP, and ATP (reactions III and I) and by measuring ADP formed in the presence of ATP and UDP (reaction III) by the method of Colowick (5). Subsequent work revealed, however, that the A. S. preparation contained myokinase and nucleoside monophosphate kinase which prevented conclusive studies on this phase of the reaction until more purified enzymes are prepared.

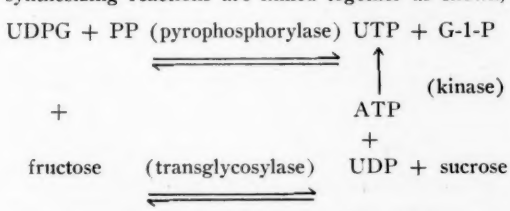
TABLE VIII

INFLUENCE OF ATP ON UDPG RESYNTHESIS AND SUCROSE SYNTHESIS BY ENZYME PREPARATIONS FROM PEA SEEDS

| EXPERIMENT | TIME (HRS) | | | | | 4 |
|---|---|---|---|---|---|---|
| | ¼ | ½ | 1 | 2 | 4 | |
| | *UDPG (μmoles/ml digest)** | | | | | *Sucrose (μmoles/ml digest)* |
| 1 +ATP | ... | ... | 0.32 | 0.29 | 0.31 | 1.5 |
| 2 +ATP | 0.48 | 0.41 | 0.31 | 0.34 | 0.45 | 1.7 |
| 1 —ATP | ... | ... | 0 | 0 | 0 | 0.9 |
| 2 —ATP | 0.25 | 0.12 | 0.04 | 0 | 0 | 0.9 |

* Reaction mixture. See Materials and Methods.

The data indicate that the UDPG and the sucrose synthesizing reactions are linked together as shown,




The enhancement by boron of sucrose synthesis as observed in the experiments with pea and sugar

cane seedling homogenates, was related evidently to the influence of the element on UDPG pyrophosphorylase and perhaps on UDP kinase. The observation that the addition of boron to the A. S. preparation does not result in an increased sucrose synthesis from UTP, G-1-P, and fructose is probably related to the difference in the levels of UDPG pyrophosphorylase and UDPG transglycosylase. The former enzyme is present in the A. S. preparation at a level approximately 100 times as great as the latter enzyme. The enzyme levels in the homogenates were not measured. Further understanding of this effect of boron on UDPG pyrophosphorylase must await purification of the enzyme.

## Summary

Boron has been observed to enhance the biosynthesis of sucrose by sugar cane and pea seedling homogenates. With an enzyme preparation from pea seeds containing UDPG pyrophosphorylase, UDPG transglycosylase, and UDP kinase boron inhibited the synthesis of sucrose from UTP, G-1-P, and fructose. In this coupled reaction ATP was essential and boron in some way caused the concentration of UDPG to be maintained; however, UDPG pyrophosphorylase and UDPG transglycosylase were inhibited by ATP when assayed individually. UDPG pyrophosphorylase was enhanced by boron while UDPG transglycosylase was inhibited.

## Literature Cited

1. Bean, R. C. and W. Z. Hassid. 1955. Synthesis of disaccharides with pea preparations. Jour. Amer. Chem. Soc. 77: 5737–5738.
2. Berg, P. and W. K. Joklik. 1954. Enzymatic phosphorylation of nucleoside diphosphates. Jour. Biol. Chem. 210: 657–672.
3. Burma, D. P. and D. C. Mortimer. 1956. The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaf. Arch. Biochem. Biophys. 62: 16–28.
4. Cardini, C. E., L. F. Leloir, and J. Chiriboga. 1955. The biosynthesis of sucrose. Jour. Biol. Chem. 214: 149–155.
5. Colowick, S. P. 1957. Adenylate kinase (myokinase, ADP phosphomutase). In: Methods in Enzymology II, S. P. Colowick and N. O. Kaplan, eds. Pp. 598–604. Academic Press, Inc., New York.
6. Dryer, R. L., A. R. Tammes, and J. Routh. 1957. The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. Jour. Biol. Chem. 225: 177–183.
7. Dugger, W. M., T. E. Humphreys, and Barbara Calhoun. 1957. The influence of boron on starch phosphorylase and its significance in translocation of sugars in plants. Plant Physiol. 32: 364–370.
8. Ganguli, N. C. 1958. Enzymatic formation of uridine diphosphate glucose with preparations from *Impatiens holstii*. Jour. Biol. Chem. 232: 337–345.
9. Gauch, H. G. and W. M. Dugger. 1953. The role of boron in the translocation of sucrose. Plant Physiol. 28: 457–466.
10. Gauch, H. G. and W. M. Dugger. 1954. The physiological action of boron in higher plant : A review and interpretation. Agr. Exp. Sta., Maryland Tech. Bull. A–80.
11. Ginsburg, V. 1958. Purification of uridine diphosphate glucose pyrophosphorylase from mung bean seedlings. Jour. Biol. Chem. 232: 55–61.
12. Hatch, M. D. and J. F. Turner. 1958. Glycolysis by an extract from pea seeds. Biochem. Jour. 69: 495–501.
13. Heppell, L. A. and R. J. Hilmoe. 1951. Purification of yeast inorganic pyrophosphatase. Jour. Biol. Chem. 192: 87–94.
14. Leloir, L. F. and C. E. Cardini. 1955. The biosynthesis of sucrose phosphate. Jour. Biol. Chem. 214: 157–165
15. Munch-Petersen, A. 1955. Investigation of the properties and mechanism of the uridine diphosphate glucose pyrophosphorylase reaction. Acta Chem. Scand. 9: 1523–1536.
16. Najjar, V. A. 1948. The isolation and properties of phosphoglucomutase. Jour. Biol. Chem. 175: 281–290.
17. Nelson, C. D. and P. R. Gorham. 1957. Uptake and translocation of $C^{14}$-labelled sugars applied to primary leaves of soybean seedlings. Can. Jour. Bot. 35: 339–347.
18. Nelson, N. 1944. A photometric adaptation of the Somogyi method on the determination of glucose. Jour. Biol. Chem. 153: 375–380.
19. Nikiforuk, G. and S. P. Colowick. 1955. 5′-Adenylic acid deaminase from muscle. In: Methods in Enzymology. II. S. P. Colowick and N. O. Kaplan, eds. Pp. 469–473. Academic Press Inc., New York.
20. Paladini, A. C. and L. F. Leloir. 1952. Studies on uridine-diphosphate-glucose. Biochem. Jour. 51: 426–430.
21. Pandy, K. P. and C. V. Ramakrishnan. 1958. Biosynthesis of sucrose in sugar cane leaves. Naturwiss. 43: 85.
22. Perkins, H. J. 1957. Chemistry of Boron in Plants. Ph D. thesis. Iowa State College, Ames.
23. Roe, J. H. 1934. A colorimetric method for the determination of fructose in blood and urine. Jour. Biol. Chem. 107: 15–22.
24. Sisler, E. C., W. M. Dugger, and H. G. Gauch. 1956. The role of boron in the translocation of organic compounds in plants. Plant Physiol. 31: 11–17.
25. Strominger, J. L., Elizabeth S. Maxwell, and H. M. Kalckar. 1957. Determination of UDPG and UTP by means of UDPG dehydrogenase. I. Enzymatic determination of UDPG. In: Methods in Enzymology III. S. P. Colowick and N. O. Kaplan, eds. Pp. 974–976. Academic Press, Inc., New York.
26. Turner, J. F. 1953. Synthesis of sucrose by a plant enzyme extract. Nature 172: 1149–1150.
27. Turner, J. F. 1957. The biosynthesis of sucrose. Biochem. Jour. 67: 450–456.
28. Turner, Donella H. and J. F. Turner. 1958. Uridine diphosphoglucose pyrophosphorylase of pea seeds. Biochem. Jour. 69: 448–452.
29. Whistler, M. E., L. Hough, and J. W. Hylin. 1953. Determination of D-glucose in corn syrups by use of glucose dehydrogenase. Anal. Chem. 25: 1215–1216.

# ROLE OF CAROTENOIDS IN PROTECTING CHLOROPHYLL FROM PHOTODESTRUCTION [1, 2]

I. C. ANDERSON AND D. S. ROBERTSON

DEPARTMENTS OF AGRONOMY AND BOTANY, AND DEPARTMENT OF GENETICS, IOWA STATE UNIVERSITY, AMES

There have been numerous suggestions in the last few years of obligatory functions of carotenoids in photosynthetic organisms. These have been functions of carotenoids other than their action as accessory pigments. Workers who are interested in the mechanism of converting radiant energy into chemical energy frequently use the carotenoid molecule as a sink, or hole, in their photodissociation hypotheses (3, 12). Kohl (9) suggested many years ago that a function of carotenoids was to protect chlorophyll. Recently, evidence for such a role of carotenoids has been accumulating. Claes (2) has described four X-ray mutants of *Chlorella vulgaris* Beyerinck. Three of these mutants do not form colored carotenoids and they are photosensitive. Sager and Zalokar (13) have studied a mutant of *Chlamydomonas rienhardi* Dang. which contains only trace amounts of carotene and which dies when exposed to light. It responds, however, to illumination by taking up carbon dioxide and releasing oxygen. Allen (1) also has shown that a carotenoidless mutant of *Chlorella pyrenoidosa* Chick is unable to grow in strong light.

The studies of Cohen-Bazire and Stanier (4) and Fuller and Anderson (6) have shown that colored carotenoids are required for protecting bacteriochlorophyll from photodestruction in the photosynthetic bacteria. It has been proposed that this is a unique role of carotenoids in all photoautotrophs (17). The results in the present communication support this hypothesis by extending to higher plants an experimental confirmation of the proposal through the study of an albino mutant of corn (white-3). Koski and Smith (10) have shown that this mutant forms protochlorophyll and chlorophyll; however, the chlorophyll is bleached upon continued illumination. An albino mutant of sunflower which reacts similarly has been described by Wallace and Schwarting (18).

## MATERIALS AND METHODS

Plant materials used in these studies were grown under three different conditions. For carotene precursor studies, white-3 and normal corn seedlings were grown on a greenhouse bench for approximately ten days. Leaf material was ground in a mixture of acetone and hexane, and a hexane fraction containing carotene and colorless precursors of carotene was isolated by the method of Zscheile and Porter (20). Spectra of these fractions were measured with a Beckman DU spectrophotometer. Hexane was passed through a dry silica gel column to decrease absorbance in the ultraviolet by reagent grade hexane.

For the photostability of chlorophyll studies, the seedlings were grown in darkness for 8 to 12 days. Each determination consisted of six whole plants which were placed in 250 ml suction flasks. The flasks were evacuated in the dark two times; after each evacuation they were filled with either air or nitrogen. The plants in the flasks were illuminated with 1500 ft-c of light produced by a bank of tungsten filament lamps with an 8 inch water filter. After the exposure period, leaves of the plants were removed, weighed, and ground immediately in a mortar with 80 % acetone and a bit of sand. Ten ml of 80 % acetone was used for each gram of leaf tissue. Optical densities of the extracts were read at 665 mμ for chlorophyll and 630 mμ for protochlorophyll.

For some experiments the seedlings were grown in dim light with an intensity of less than 0.5 ft-c. Under these conditions, the white-3 seedlings were a blue-green color, and the normal seedlings a yellow-green. Both types of seedlings could be grown for two to three weeks under these conditions before they died.

## RESULTS

ACCUMULATION OF PHYTOENE BY WHITE-3: The visible and ultraviolet spectra of the hexane extracts of normal and white-3 seedlings are presented in figure 1. The white-3 seedlings did not contain colored carotenoids as did normal seedlings. Instead, they had large amounts of substance which absorbed light in the ultraviolet region with peaks at 275, 285, and 297 mμ. These peaks are similar to those of phytoene (7), a precursor of colored carotenoids. Ergosterol, which has a spectrum similar to that of phytoene (7), was added during homogenization of white-3 corn seedlings, and after fractionation the hexane extract was poured through a neutral alumina column. The ultraviolet absorbing substance of the seedlings (phytoene) came through the column in the first 5 ml fraction, whereas added ergosterol appeared in the sixth 5 ml fraction when the column was leached with hexane.

[1] Received manuscript December 2, 1959.

[2] Journal Paper No. J-3767 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project No.'s 1423 and 1381.

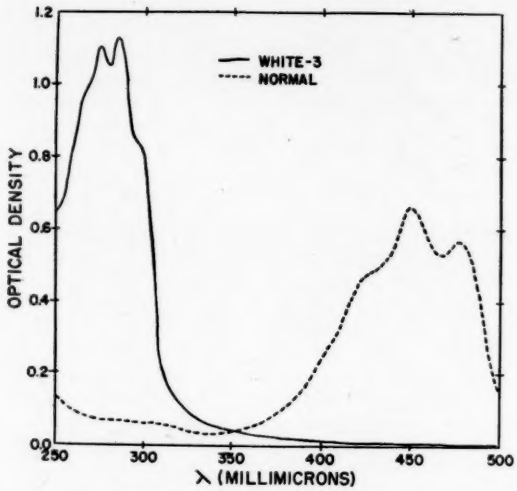
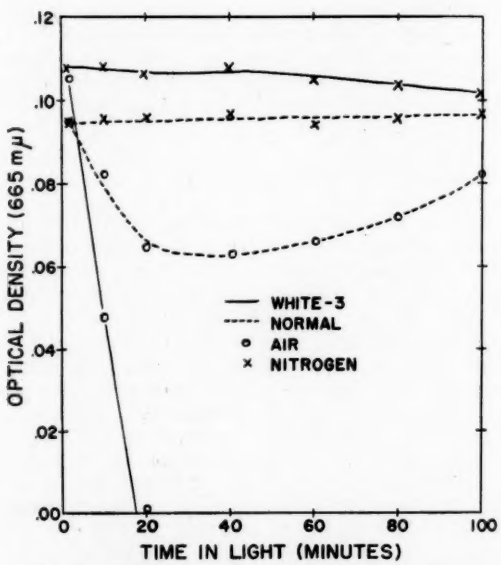



FIG. 1. Spectrum of hexane extracts from normal and white-3 seedlings.

PHOTOSTABILITY OF CHLOROPHYLL IN SEEDLINGS: As reported by Koski and Smith (10), dark grown mutant seedlings contained at least as much, usually more, protochlorophyll than did normal seedlings. The protochlorophyll of both types of genetic materials was readily converted to chlorophyll upon exposure of the seedlings to light. The results of exposing mutant and normal seedlings to light under various conditions are reported in figure 2. Chlorophyll of the mutant was completely destroyed when seedlings were illuminated in an atmosphere of air. Under anaerobic conditions, however, chlorophyll of the mutant was stable to high light intensities.

When leaves of normal seedlings were illuminated in air there was an initial decrease in chlorophyll content for the first 20 minutes, then a stabilization, and eventually an increase in chlorophyll content. It was suspected that this phenomenon might parallel the conversion of chlorophyllide a to chlorophyll a. Wolff and Price (19) have shown, in beans, that protochlorophyllide is photochemically reduced to chlorophyllide a, and that chlorophyllide a is esterified with phytol to form chlorophyll a in subsequent longer dark periods. Also with regard to this phenomenon, a study made by Granick (8) indicates a greater photolability of chlorophyllide a than of chlorophyll a. Some experiments were conducted with the corn seedlings, using the differential solubility of chlorophyllide and chlorophyll in the binary system of petroleum ether and acetone (19). These showed that most of the pigment which was formed by 1 minute of illumination was chlorophyllide; in a subsequent longer dark period the chlorophyllide was esterified to form chlorophyll. Nearly all the pigment from



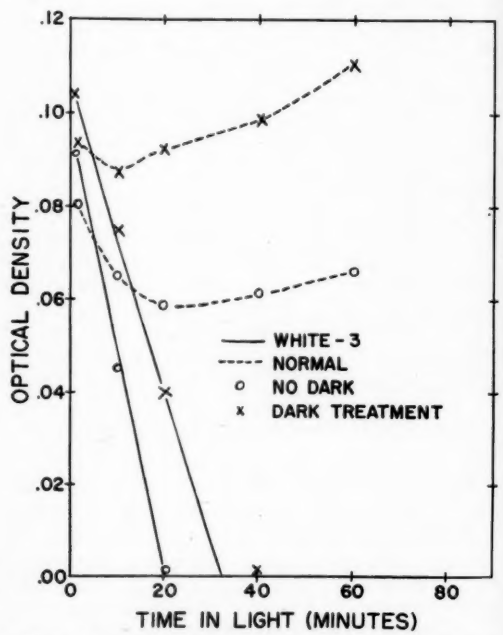




FIG. 2. Stability of chlorophyll of normal and white-3 seedlings as affected by exposure to light under aerobic and anaerobic conditions.

FIG. 3. Stability of chlorophyll a (dark treatment) and chlorophyllide a (no dark) of seedlings exposed to light in air.

both normal and mutant seedlings could be transferred to the petroleum ether phase after 30 to 40 minutes of darkness.

Since the previous experiments demonstrated that most of the pigment formed after 1 minute of illumination was chlorophyllide and that this was converted to chlorophyll during the dark treatment, any difference in the stability of pigment content to light in plants treated so as to contain mainly chlorophyllide or chlorophyll can be used to test the stability of these two forms of chlorophyll. Dark grown seedlings were illuminated for 1 minute and then either placed in the dark for 1 hour before being re-exposed to light or given no dark treatment and immediately exposed to further light. Pigment (chlorophyllide) in seedlings without a dark treatment was much less stable than the pigment (chlorophyll) in seedlings that had a dark treatment (fig 3). This was the most prominent during the first few minutes of illumination of normal seedlings. These results suggest that the non-esterified form of chlorophyll is less stable than the esterified form.

The continuous formation of protochlorophyll during the dark period after a brief illumination can be measured. It is illustrated also in figure 3 by the greater chlorophyll content of the plants receiving the dark treatment than those which did not receive the dark treatment. In fact, the small decrease in chlorophyll content during the first 10 minutes of illumination of the normal seedlings which had received the dark treatment probably represents a photochemical destruction of chlorophyllide a formed from the amount of protochlorophyllide which had accumulated during 1 hour of darkness.

It is of interest to consider the effect of anaerobic conditions on the formation of protochlorophyll and chlorophyll at the seedling stage of development. Anaerobiosis did not inhibit the conversion of preformed protochlorophyll to chlorophyll (fig 2). This has been reported previously by Smith (15). In this same figure, it is apparent that under anaerobic conditions there was no appreciable increase in the chlorophyll content of normal seedlings, whereas under aerobic conditions there was an appreciable increase in chlorophyll content between 40 and 100 minutes. The implication is that the formation of protochlorophyll requires oxidative metabolism. This is not unexpected if one considers that the citric acid cycle is the source of intermediates and energy for porphyrin synthesis (14).

Seedlings Grown With Dim Light: White-3 and normal seedlings were grown under a light intensity of less than 0.5 ft-c. This light intensity allowed for a continual increase in chlorophyll content over the 2 week period (table I). After 2 weeks of growth the seedlings were exposed to bright light for 1 hour in air. Nearly all the chlorophyll of the white-3 seedlings was destroyed by this treatment, whereas the chlorophyll of the normal seedlings was not appreciably effected by bright light.

A green pellet could be sedimented by 1000 × G from both normal and mutant materials when leaves were ground with sand in 0.35 M NaCl. Under the microscope, chloroplasts could be recognized readily in the pellet from the normal seedlings. The chloroplast-like particles in the pellet from mutant seedlings were rather opalescent but otherwise appeared to be normal. Leaves of the two types of seedlings were embedded in paraffin and sectioned. The chloroplasts of the mutant appeared to be as numerous and of the same size as those of the normal seedlings. Further studies are in progress on the number and size of chloroplasts and the capacity of chloroplasts from mutant seedlings to carry out the reactions of photosynthesis.

Table I

Accumulation of Chlorophyll by Seedlings Grown Under Dim Light and Its Stability to Bright Light

| Seedling | Dim light | | Bright light for 1 hr after 14 days dim light |
|---|---|---|---|
| | 7 Days | 14 Days | |
| | *μg chlorophyll/g fresh weight* | | |
| Normal | 19 | 61 | 58 |
| White-3 | 12 | 28 | 5 |

## Discussion

In this particular albino mutant of corn, chlorophyll and chloroplast formation appear to be normal. That is, protochlorophyllide of dark grown mutant seedlings is converted to chlorophyllide by short exposure to light. The mutant tissue has the capacity to esterify chlorophyllide to form chlorophyll. If grown under dim light conditions chloroplast development in the mutant appears to be similar to that of normal seedlings.

Chlorophyll of the mutant, however, is unstable to high light intensities in the presence of oxygen. Under strictly anaerobic conditions the chlorophyll of the mutant is stable to strong light. These results are similar to those reported for the carotenoidless condition in photosynthetic bacteria (4, 6). As in the bacteria, the instability of chlorophyll appears to be due to the lack of colored carotenoids in the mutant. These results, then, support Stanier's (17) prediction that colored carotenoids are required in all aerobic photoautotrophs for the protection of chlorophyll from autophotodestruction.

The function of carotenoid pigments for protection of chlorophyll from photodestruction may not be limited to photoautotrophs. It may have far wider significance than this. Eyster (5) has shown that the catalase activity of an albino mutant of corn is considerably lower than it is in normal seedlings. Mathews and Sistrom (11) recently showed that cells of a carotenoidless mutant of *Sarcina lutea* Schroeter are killed when placed in sunlight under aerobic conditions. Nearly all air borne pollen grains

and many air borne fungal spores contain carotenoids. It is entirely conceivable that carotenoids may protect other light absorbing porphyrins such as catalase and cytochrome from photodestruction.

We do not wish to imply that all albino mutants are caused by a metabolic block in carotenoid biosynthesis. There presumably is a different metabolic block in all the nonallelic albino mutants of a species. Consequently, there are many possible causes of albinism. For example, a metabolic abnormality that prevented normal chloroplast development could cause albinism. The studies of Smith (16) show, however, that many of the albino mutants of corn lack carotene. Our preliminary survey of the same group of mutants indicates that there are a number of metabolic blocks at different steps in the synthesis of carotenoids.

Carotenoids conceivably may have more than one obligatory role in higher plants, but a basic function is the protection of chlorophyll from photooxidation. In the obligately anaerobic photosynthetic bacteria there is no such strict requirement of carotenoids for the protection of chlorophyll from destruction, or for any other obligate role, since carotenoidless bacteria grow equally as well as do those containing carotenoids (4). In the aerobic photoautotrophs, on the other hand, the presence of colored carotenoids is vital for autotrophic growth in the light. Other obligatory roles of carotenoids may have evolved in higher plants along with the evolution of the more complex photosynthetic system of the chloroplast, in which oxygen is produced, compared to the non-oxygen producing chromatophore of the bacteria. The albino mutants seem to be very useful tools for further studies on the function of carotenoid pigments in photosynthesis.

## Summary

There is a metabolic block between phytoene and colored carotenoid synthesis in white-3. Chlorophyll and chloroplast formation by the mutant appears to be normal, but its chlorophyll is destroyed by high light intensities under aerobic conditions. Under anaerobic conditions, however, the chlorophyll of the mutant is stable to strong light. The results support the thesis that an important role of colored carotenoids is the protection of chlorophyll from photodestruction.

## Acknowledgment

This work was supported in part by a grant from the National Science Foundation (G–6452). We gratefully acknowledge this support.

## Literature Cited

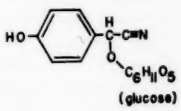
1. Allen, M. B. 1959. Possible functions of chlorophyll b. Studies with green algae that lack chlorophyll b. In: The Photochemical Apparatus, Its Structure and Function. Brookhaven Symposia in Biology. No. 11, Pp. 339–342.
2. Claes, H. 1954. Analyse der biochemischen Synthesekette für Carotinoide mit Hilfe von Chlorella-Mutanten. Zeit. Naturforscher 9b: 461–469.
3. Calvin, M. 1959. From microstructure to macrostructure and function in the photochemical apparatus. In: The Photochemical Apparatus, Its Structure and Function. Brookhaven Symposia in Biology. No. 11, Pp. 160–180.
4. Cohen-Bazire, G. and R. Y. Stanier. 1958. Specific inhibition of carotenoid synthesis in a photosynthetic bacterium and its physiological consequences. Nature 181: 250–252.
5. Eyster, C. 1950. Catalase activity in chloroplast pigment deficient types of corn. Plant Physiol. 25: 630–638.
6. Fuller, R. C. and I. C. Anderson. 1958. Suppression of carotenoid synthesis and its effect on the activity of photosynthetic bacterial chromatophores. Nature 181: 252–254.
7. Goodwin, T. W. 1952. Studies in carotenogenesis 3. Identification of the minor polyene components of the fungus *Phycomyces blakesleeanus* and a study of their synthesis under various conditions. Biochem. Jour. 50: 550–558.
8. Granick, S. 1950. Magnesium vinyl pheoporphyrin $a_5$, another intermediate in the biological synthesis of chlorophyll. Jour. Biol. Chem. 183: 713–730.
9. Kohl, F. G. 1902. Untersuchungen über das Carotin und sein physiologische Bedeutung in der Pflanze. Pp. 9–11. Gebruder Borntraeger, Berlin.
10. Koski, V. M. and J. H. C. Smith. 1951. Chlorophyll formation in a mutant, white seedling-3. Arch. Biochem. Biophys. 34: 189–195.
11. Mathews, M. and W. R. Sistrom. 1959. Function of carotenoid pigments in non-photosynthetic bacteria. Nature 184: 1892–1893.
12. Platt, J. R. 1959. Carotene-donor-acceptor complexes in photosynthesis. Science 129: 372–374.
13. Sager, R. and M. Zalokar. 1958. Pigments and photosynthesis in a carotenoid-deficient mutant of Chlamydomonus. Nature 182: 98–100.
14. Shemin, D. and C. S. Russell. 1953. δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. Jour. Amer. Chem. Soc. 75: 4873–4874.
15. Smith, J. H. C. 1954. The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. Plant Physiol. 29: 143–148.
16. Smith, J. H. C., L. J. Durham, and C. F. Wurster. 1959. Formation and bleaching of chlorophyll in albino corn seedlings. Plant Physiol. 34: 340–345.
17. Stanier, R. Y. 1959. Formation and function of photosynthetic pigment system in purple bacteria. In: The Photochemical Apparatus, Its Structure and Function. Brookhaven Symposia in Biology. No. 11, Pp. 43–53.
18. Wallace, R. H. and A. E. Schwarting. 1954. A study of chlorophyll in a white mutant strain of *Helianthus annuus*. Plant Physiol. 29: 431–436.
19. Wolff, J. B. and L. Price. 1957. Terminal steps of chlorophyll a biosynthesis in higher plants. Arch. Biochem. Biophys. 72: 293–301.
20. Zscheile, F. P. and J. W. Porter. 1947. Analytical methods for carotenes of Lycopersicon species and strains. Ind. Eng. Chem., Anal. Ed. 19: 47–51.

# STUDIES ON CYANOGENIC GLYCOSIDE OF SORGHUM VULGARE [1, 2]

T. AKAZAWA [3], P. MILJANICH [4], AND ERIC E. CONN

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, DAVIS

The cyanogenic glycoside dhurrin (*p*-hydroxymandelonitrile-*β*-D-glucoside[5]) occurs in members of the Sorghum family. Dunstan and Henry, who first




isolated and characterized the compound from *Sorghum vulgare* Pers., reported that the glycoside was abundant in young plants but was absent in mature plants and in the seed (5). These observations suggested that this tissue might be suitable for investigating the biosynthesis of *p*-hydroxybenzaldehyde and HCN. This paper describes experiments designed to test the usefulness of *Sorghum vulgare* as an experimental material and defines the conditions used in subsequent biosynthetic studies (4).

## MATERIALS AND METHODS

The experiments were carried out with *Sorghum vulgare* (var. Honey Drip) purchased from Germain's Inc., Fresno. In preliminary experiments, 3-day old etiolated seedlings of this variety contained more HCN than ten other varieties examined. Green seedlings were grown at 25° C in vermiculite in metal trays from seed which had been soaked for 16 hours in tap water. The trays were placed under three fluorescent tubes (40 w each) and light was supplied for 16 hours daily. The material obtained under these conditions was more uniform than that grown in the greenhouse in daylight. Only the aerial portion of the seedlings was taken for analysis; 25 to 30 seedlings weighed 1 g.

Etiolated seedlings were obtained essentially as described by Jacobson et al (8). Seeds which had been soaked for 16 hours in tap water were spread on moist cheese cloth mounted on inverted, perforated, plastic baskets. The baskets were placed in a 4 liter beaker containing 1 liter of dilute mineral solution (8) so that the seeds were supported about 0.5 cm above the surface of the solution. The beaker was then covered with a watch glass, placed in a light-proof box at 30° C, and the solution aerated with a tube extending into the beaker. Under these conditions extremely uniform germination and growth occurred and, after 3 days, the roots were 6 to 7 cm in length while the shoots were 9 to 10 cm. The complete seedling was taken for analysis; 10 to 15 seedlings weighed 1 gram.

In most of the work reported here, no attempt was made to isolate the cyanogenic glycoside quantitatively. Instead, because of the occurrence of an active glycosidase in sorghum (5), it seemed desirable to permit this enzyme to catalyse the hydrolysis of *p*-HMN-*β*-G and then estimate the glycoside by measuring the HCN and *p*-hydroxybenzaldehyde released. Upon hydrolysis of the glucoside, the aglycone formed is *p*-hydroxymandelonitrile. This compound is unstable and decomposes to HCN and the aromatic aldehyde. In addition, however, an enzyme (oxynitrilase (1)), which catalyzes the decomposition of the nitrile, is also present in sorghum (4). A commercial preparation of emulsin (Worthington Biochemicals) also catalyzed the decomposition of *p*-HMN-*β*-G to HCN, *p*-hydroxybenzaldehyde and glucose. Acid hydrolysis of the glycoside was carried out in 1.4 *N* $H_2SO_4$ at 80° C for 5 hours.

The plant material was prepared for analysis by freezing in liquid nitrogen, then, grinding to a fine powder with a mortar and pestle. The powder was suspended in water (50 ml/g) and air was passed through the homogenate into 10 ml of 0.1 *N* NaOH to trap HCN produced during hydrolysis of the *p*-HMN-*β*-G. During the aeration period, aliquots of the homogenates were removed and mixed with equal volumes of 10 % trichloroacetic acid and centrifuged to provide a clear supernatant solution. If emulsin or $H_2SO_4$ was used to hydrolyze the glycoside, these substances were added and the reaction mixture was aerated to remove and trap any HCN formed.

Analyses for HCN were usually done by the method of Liebig. The HCN content of the 0.1 *N* NaOH trapping solution (10 ml) was determined directly by adding 2.0 ml 20 % $NH_4Cl$ and three drops 5 % KI to the NaOH, then titrating with 0.002 M $AgNO_3$. All readings were corrected for a blank of approximately 0.12 ml. In some instances HCN was also determined by the method of Aldridge (2). *p*-Hydroxybenzaldehyde in the 5 % trichloroacetic acid supernatant was analysed as the 2,4-dinitrophenylhydrazone (6). Since *p*-hydroxybenzaldehyde strongly absorbs light at 330 mμ in 0.1 *N* NaOH (E = 27,300), the aldehyde was also analysed by

---

[1] Manuscript received December 16, 1959.

[2] This study was supported in part by a research grant (RG–5301) from the National Institutes of Health, United States Public Health Service.

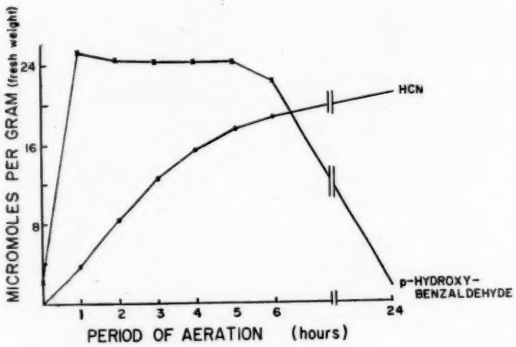
[3] Present address, Faculty of Agriculture, Nagoya University, Anjo, Aichi-ken, Japan.

[4] Present address, Department of Home Economics, University of California, Berkeley.

[5] *p*-Hydroxymandelonitrile-*β*-D-glucoside is abbreviated as *p*-HMN-*β*-G.

placing aliquots of the 5 % trichloroacetic acid supernatant solution in 0.1 *N* NaOH and measuring the light absorption at 330 mμ. This procedure gave good agreement with the 2,4-dinitrophenylhydrazone procedure because the concentration of the aldehyde in the plant material far exceeded that of any other material which absorbed light under the described conditions. In 3-day old seedlings, the *p*-hydroxybenzaldehyde was approximately 0.25 % of the wet weight of the tissue.

The studies on alkaline instability of *p*-HMN-β-G were carried out on an aqueous solution of glycoside obtained by extracting etiolated sorghum seedlings with 80 % ethanol. The alcoholic extract was concentrated and chromatographed in 1-butanol : pyridine : water (6 : 4 : 3) (*7*). The glycoside (Rf=0.85) was then eluted with $H_2O$ and the eluate in turn extracted three times with equal volumes of diethyl ether to remove any *p*-hydroxybenzaldehyde. After aeration to remove the dissolved ether, the aqueous fraction was used in the studies on decomposition of the glycoside by alkali.



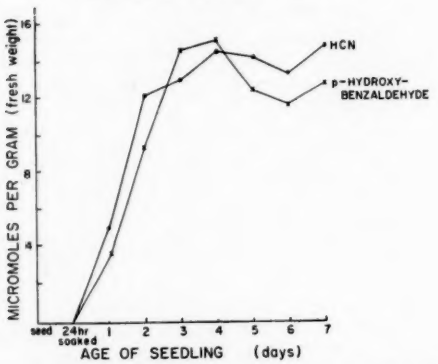




FIG. 1. The enzymatic hydrolysis of *p*-hydroxymandelonitrile-β-D-glucoside in homogenates of etiolated seedlings. One gram of plant material (15 seedlings) was used.

FIG. 2. The cyanogenic glycoside content of etiolated sorghum seedlings of different ages. In these experiments, *p*-hydroxybenzaldehyde was determined as the 2,4-dinitrophenylhydrazone and HCN was measured by titration with $AgNO_3$. One gram of plant material was analyzed.

## RESULTS AND DISCUSSION

Figure 1 shows the results obtained when 3-day old etiolated seedlings were ground and suspended in $H_2O$ and analyses were performed for *p*-hydroxybenzaldehyde at various intervals. There was only a negligible amount of aldehyde present in a sample taken within 3 minutes after homogenization. Within 1 hour, however, the aldehyde content reached its maximum value and remained constant for 6 hours.

The removal of HCN by aeration of the homogenized plant material is also indicated in figure 1. Although one would expect an amount of HCN equal to the *p*-hydroxybenzaldehyde to be present after 1 hour of enzymatic digestion, the aeration procedure used was not sufficiently rapid to remove all of the HCN released during that period. Maximum values for the HCN content of the homogenate were not obtained except after aeration for 16 to 24 hours. The data in figure 1 do show however that approximately equimolar quantities of HCN and aldehyde were produced. Therefore, in these studies the aldehyde content was determined at 2 or 3 hours, while the HCN analysis was carried out after aeration of the homogenate for 16 hours.

When aeration of the homogenate was continued over night to remove HCN formed during the decomposition of *p*-HMN-β-G, the aldehyde content of the solution decreased sharply (fig 1). The reaction responsible for the disappearance of *p*-hydroxybenzaldehyde is not known. The reaction is enzymatic however, and since it requires aeration, it probably is aerobic. The disappearance of *p*-hydroxybenzaldehyde did not result in the formation of *p*-hydroxybenzoic acid nor a compound capable of reacting with 2,4-dinitrophenylhydrazine.

Figure 2 presents data for the *p*-hydroxybenzaldehyde and HCN content of etiolated seedlings of different ages. The amounts of the two compounds are equal and reach maximum values after 3 days. Since there was little *p*-hydroxybenzaldehyde in these plants when the tissue was first homogenized and since an amount of HCN equivalent to the aldehyde was formed, the results also indicate that both the HCN and aromatic aldehyde were present initially only as components of the cyanogenic glycoside. The amount of HCN or *p*-hydroxybenzaldehyde produced corresponds therefore to the *p*-HMN-β-G in the seedling. The etiolated plant material could not be maintained in a healthy state beyond 7 days and measurements were discontinued at that point.

Figure 3 presents the amounts of HCN and *p*-hydroxybenzaldehyde produced as a result of enzymatic hydrolysis of *p*-HMN-β-G in the aerial portion of green sorghum seedlings of different ages. The data show that the two compounds again were present in approximately equal concentrations throughout the period examined (24 days). In contrast to the

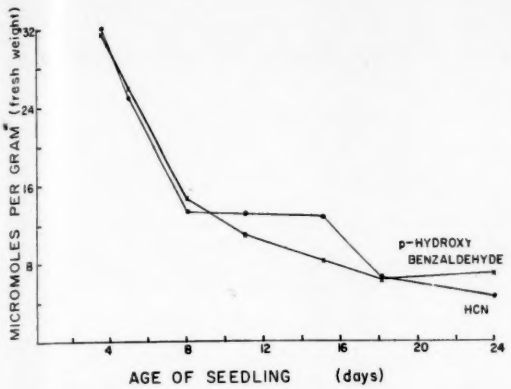



FIG. 3. The cyanogenic glycoside content of green sorghum seedlings of different ages. Analyses were the same as for figure 2.

etiolated seedling, the concentration of the aldehyde and HCN decreased as the seedling increased in age. This observation agrees with that of Dunstan and Henry (5) who reported that mature plants lacked the glycoside. The HCN content of the green plants is also in agreement with those (0.2 % dry wt) reported by the earlier workers (5).

In preliminary studies of the *p*-HMN-*β*-G content of green seedlings, no agreement between the HCN and aldehyde formed could be observed when homogenates of the older plants were analysed for *p*-hydroxybenzaldehyde at 3 hours. Instead maximum values for the aldehyde were obtained much earlier during the digestion and, at 3 hours, the destruction and disappearance of *p*-hydroxybenzaldehyde, previously described in etiolated seedlings (fig 1), was already far advanced. Therefore, the aldehyde content of the homogenates of green seedlings was determined at 30 minute intervals after homogenization, and the maximum values observed were compared with the HCN obtained on aeration. The time required to obtain the maximum value of *p*-hydroxybenzaldehyde varied with the age of the green seedling. In the case of the youngest plants in figure 3, the maximum value was reached only after 3 hours of digestion while, with the oldest plants, the maximum value was reached in 30 minutes.

In comparing the *p*-HMN-*β*-G content of etiolated and green seedlings, it should be emphasized that only the aerial portion of the green seedling was analyzed. Since this portion constituted about half the weight of these seedlings, the glycoside contents of the etiolated and green seedlings are approximately equal. Root development of the etiolated seedlings was extensive at 3 days, however, and the growth of this type of seedling was much more uniform than the growth of green seedlings. Since the proposed biosynthetic studies required the feeding of radioactive precursors through the roots of the experimental plants, the following observations were made on the nature of the glycoside as it occurs in etiolated seedlings.

Although the data in figure 1 indicated that little or no free HCN or *p*-hydroxybenzaldehyde existed in the sorghum seedling prior to enzymatic hydrolysis of *p*-HMN-*β*-G, it was important to investigate this point more thoroughly. This was done by extracting twenty 3-day old etiolated seedlings (1.39 gm) with 30 ml of 90 % ethanol and aerating the ethanolic supernatant to remove and measure HCN. Under these conditions only a trace of HCN was found. The ethanolic extract was then concentrated to dryness and the residue was taken up in 30 ml of $H_2O$. Further aeration of this aqueous solution produced no additional HCN. However, when 51 mg of an emulsin preparation was added and digestion and aeration continued for 16 hours, 21.0 μmoles of HCN per gram of seedling was produced. This value corresponds to the glycoside content of seedlings of this age (fig 2) and indicates that no hydrolysis of *p*-HMN-*β*-G occurred until emulsin was added to the residue of the ethanol extract. The absence of HCN in the ethanol extract shows that little or no free HCN exists in the plant.

Dry sorghum seeds were also analyzed for HCN and *p*-hydroxybenzaldehyde. Analysis of aqueous extracts of the seed showed neither of these compounds to be present. Since the seed conceivably might lack the *β*-D-glucosidase necessary for hydrolysis of the glucoside, 50 mg of emulsin was added to the aqueous extracts. Neither aldehyde nor HCN was produced under these conditions; subsequent digestion of the extract with $H_2SO_4$ at 80° C for 5 hours also failed to release HCN or *p*-hydroxybenzaldehyde.

In view of the extensive synthesis of glycoside which must be occurring in the seedling, it was of interest to determine the localization of the glycoside within the young plant. Therefore, twenty 4-day old etiolated seedlings were divided into roots (0.16 gm), seed residues (0.35 gm), and shoots (1.35 gm), and analysed for HCN. After digestion and aeration for 16 hours, the shoots contained 18.5 μmoles of HCN per gram. The roots and seed residues contained no detectable HCN and, although these tissues conceivably could lack the glycosidase necessary for enzymatic hydrolysis of the glycoside, hydrolysis with 1.4 *N* $H_2SO_4$ for 5 hours at 80° C failed to produce any HCN.

During these studies an apparent instability of *p*-HMN-*β*-G in alkaline solution was observed. When samples of the glycoside (0.05 μmoles per ml) were placed in 0.1 *N* NaOH at room temperature, the light absorption at 330 mμ increased over a period of 120 minutes. At the end of this time, the absorption spectrum of the solution was characteristic of *p*-hydroxybenzaldehyde ($E_{max}$ at 330 mμ). From the increase in light absorption the amount of *p*-hydroxybenzaldehyde apparently formed was calculated. The alkaline solution was then analyzed for substances capable of reacting with 2,4-dinitrophenylhydrazine

TABLE I

ALKALINE HYDROLYSIS OF *p*-HYDROXYMANDELONITRILE β-D-GLUCOSIDE

| | EXPT. I | EXPT. II |
|---|---|---|
| *p-Hydroxybenzaldehyde formed* | | |
| (a) Determined by light absorption at 330 mμ | 0.418 μmole | 0.747 μmole |
| (b) Determined as 2,4-dinitrophenylhydrazone | 0.375 μmole | 0.735 μmole |
| *HCN formed* | 0.465 μmole | 0.864 μmole |

Samples of the glucoside (0.4–0.75 μmoles) were added to 0.1 *N* NaOH in a final volume of 15 ml. The resulting increase in light adsorption at 330 mμ (about 0.8–1.4 O.D. units) was followed until no further increase occurred (2.5 hrs). Aliquots (2.0 ml) of the alkaline solution were then analyzed for HCN and carbonyl value. In the absence of NaOH an aqueous solution of *p*-HMN-β-G contained neither HCN nor carbonyl-reactive material after standing 2.5 hours.

and for HCN (2). The data in table I show that the amount of carbonyl reactive material determined as the phenylhydrazone was equivalent to the aldehyde content as determined from the increase in light absorption. The amount of HCN produced as a result of treatment with NaOH is also in agreement with the amount of aldehyde released.

Dunstan and Henry (5) reported that alkaline hydrolysis of *p*-HMN-β-G at elevated temperatures initially yielded dhurrinic acid. Upon subsequent hydrolysis with acid this compound formed *p*-hydroxymandelic acid and glucose. The present data indicate that, at room temperature, alkaline treatment results in hydrolysis of the glycosidic bond to yield *p*-hydroxymandelonitrile which in turn decomposes to *p*-hydroxybenzaldehyde and HCN. Alkaline labile glycosides are well known (3). Amygdalin was unaffected by 0.1 *N* NaOH under the same conditions.

## SUMMARY

A method for germinating uniform, etiolated seedlings of *Sorghum vulgare* Pers. is described. Upon homogenization, the cyanogenic glycoside which occurs in these plants is hydrolyzed enzymically and equimolar amounts of HCN and *p*-hydroxybenzaldehyde are formed. The glycoside is localized in the aerial shoots of the plant. Seeds of Sorghum do not contain the glycoside but 3-day old etiolated seedlings may contain as much as 15 μmoles per gram fresh weight. The 5-day old green seedling contains approximately the same amount of glycoside but in this plant the amount decreases as the plant increases with age. Evidence for the instability of the cyanogenic glycoside in alkaline solutions is also presented.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Maryanna Pearson and Charlotte Schmid in some of the experiments.

## LITERATURE CITED

1. ALBERS, H. 1941. Oxynitrilase. Die Methoden der Fermentforschung. E. Baumann and K. Myrbach, eds. 3: 2137–2149.
2. ALDRIDGE, W. N. 1944. A new method for the estimation of micro quantities of cyanide and thiocyanate. Analyst 69: 262–265.
3. BALLOU, C. E. 1954. Alkali-sensitive glycosides. In: Advances in Carbohydrate Chem. 9: 59–95.
4. CONN, E. E., and T. AKAZAWA. 1958. Biosynthesis of *p*-hydroxybenzaldehyde. Fed. Proc. 17: 205.
5. DUNSTAN, W. R. and T. A. HENRY. 1902. Cyanogenesis in plants II. The great millet *Sorghum vulgare*. Phil Trans, Royal Soc. (London) Ser. A. 199: 399–410
6. FRIEDEMANN, T. E., and G. E. HAUGEN. 1943. Pyruvic acid II. The determination of keto acids in blood and urine. Jour. Biol. Chem. 147: 415–442.
7. GANDER, J. E. 1960. Factors influencing in vivo formation of *p*-hydroxymandelonitrile-β-D-glucoside. Plant Physiology: (In press)
8. JACOBSON, L., R. OVERSTREET, H. M. KING, and R. HANDLEY. 1950. A study of potassium absorption by barley roots. Plant Physiol. 25: 639–647.

# CHANGES IN CERTAIN NITROGENOUS CONSTITUENTS OF WINTER WHEAT AS RELATED TO COLD HARDINESS [1, 2]

A. W. PAULI AND H. L. MITCHELL [3]

Lack of sufficient cold hardiness has long been a problem in the stabilized production of crop plants in many areas of the world. While notable progress has been made through breeding and selecting hardier types, much remains to be learned regarding the mechanism by which plants become hardy and the factors responsible for differences in hardiness among groups of plants. Various biochemical changes, particularly of the carbohydrates (8), have been shown to be associated with cold hardiness. Recent findings have indicated nitrogenous constituents also may be important.

Levitt (10) has published a comprehensive review of research on the relation of nitrogenous substances to cold hardiness. Data on the relation between nitrogenous compounds and hardiness have generally been conflicting. Newton (11) reported an increase in both the amino and water soluble nitrogen content of winter wheats during the fall. The hardiest variety had the highest percentage of water soluble nitrogen, but the relation was not uniform throughout the series. The percentage of crude protein did not vary greatly with hardening. Janssen (7) found that water soluble nitrogen and soluble nitrogen coagulable by heat increased in wheat during the fall as temperatures decreased. No change in total nitrogen was noted. Newton and Brown (12), however, found no relationship between nonprotein or amino nitrogen and cold hardiness in wheat; Dexter (6) found no inverse relationship in barley, oats, and cabbage.

At least a part of the confusion apparently has resulted because most workers were concerned with total nitrogen or mixtures of nitrogenous compounds rather than with individual nitrogenous substances. Briggs and Siminovitch (3), in a study of individual nitrogen fractions in the bark of locust trees, found only the water soluble protein nitrogen fraction to be significantly associated with cold hardiness. Bula et al (4) reported similar findings with alfalfa.

The purpose of the present study was to compare changes in certain nitrogen fractions in hardened and unhardened wheat plants of the same age.

## Materials and Methods

Pawnee winter wheat plants used in this study were grown in 6 inch clay pots in growth chambers. The soil used in the experiment contained adequate quantities of nitrogen for good growth of wheat. Light intensity was approximately 1300 ft-c 1 foot from the plants. A 12 hour photoperiod was maintained throughout the experiment. Temperatures were controlled within ±1° F. Temperatures were maintained at 70° F for 3 weeks following planting, after which one group of plants was hardened at 35° F for 6 weeks. It was considered possible that biochemical changes which might be detected in hardened plants might be a function of age and might be independent of hardening. Therefore, a second group of plants was continued in an unhardened condition at 70° F so that hardened and unhardened plants of the same calendar age could be compared. At the first sampling date, unhardened plants were about eight inches high. After 4 weeks of hardening (3rd sampling date), hardened plants had assumed the prostrate condition associated with prolonged exposure to low temperatures.

Plants were harvested by clipping the stems at soil level. Duplicate analyses were made at the beginning of the hardening period and at 2 week intervals thereafter for the various nitrogen fractions. Total nitrogen was determined by the AOAC Kjeldahl-Gunning method (1). Water insoluble nitrogen, water soluble protein nitrogen, and water soluble nonprotein nitrogen were determined by blending 5 g of fresh tissue with 100 ml of water in a manner outlined by Briggs and Siminovitch (3).

To determine the remaining fractions, 5 g of plant tissue and 80 ml of re-distilled water were placed in a 50 ml Sorvall Omnimixer cup and the mixer was operated at full speed (16,000 rpm) for 3 minutes. The sample was poured into a Buchner funnel, the cup and cutting assembly were rinsed repeatedly with water, and the rinsings were used to wash the pulp retained by the filter. The filtrate was diluted to 200 ml and was transferred to a 400 ml beaker. The pH was adjusted to 4.5 with glacial acetic acid by means of a Beckman pH meter. The solution was heated barely to boiling to coagulate protein, and then was cooled rapidly by placing the beaker in cold water. The cooled solution was filtered by gravity through dry filter paper, and the pH was adjusted to 7 with a few drops of 50 % sodium hydroxide,

Ammonia and amides were determined by the method of Pucher, Vickery, and Leavenworth (13).

Amino acids were estimated by the procedure of Woiwood (15), as modified by Beauchene et al (2). Five milliliters of the extract were placed in a 15 ml graduated centrifuge tube. Five milliliters of copper phosphate suspension was added, the volume was adjusted to 15 ml with water, and the contents of the tube were mixed. The mixture was centrifuged at 2000 rpm for 5 minutes to sediment the excess copper

[1] Received December 18, 1959.

[2] Contribution No. 668, Department of Agronomy and 594, Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan.

[3] Assistant Agronomist and Biochemist, respectively.

phosphate. Since the plant extracts had slight yellow coloring and often were slightly turbid, no attempt was made to determine the amino acids by measuring the intensity of the blue color produced at this stage. Instead, the complexed copper was determined by placing 2 ml of the blue solution in a 40 ml glass-stoppered centrifuge tube and adding 10 ml of water and 0.1 ml of 2% sodium diethyldithiocarbamate solution. Twenty milliliters of iso-amyl alcohol was added and the tube was shaken vigorously to transfer the yellow copper diethyldithiocarbamate to the alcohol phase. The mixture was centrifuged for 5 minutes to break the emulsion, and the absorbance of the alcohol phase was measured at 435 mμ with a Beckman DU spectrophotometer. A blank was carried through the procedure to correct for traces of copper in the extract and reagents. A standard curve was prepared by use of alanine solutions.

The degree of cold hardiness was determined by the electrical conductivity method of Dexter (5) who found there was a close association with cold hardiness. Three gram tissue samples were frozen 4 hours at 20° F and thawed for 20 hours at 35° F in 100 ml of water. After the liquid surrounding the tissue was brought to room temperature, resistance readings were made and were converted to conductivity ($ohms^{-1}$).

## Results and Discussion

From table I it is apparent that when the data were calculated on a fresh-weight basis marked differences occurred in most of the nitrogen fractions. Total nitrogen, soluble protein nitrogen, and soluble nonprotein nitrogen contents increased during hardening. The results agree with those reported previously for forage plants (4). However, when the data were expressed as percentage of total nitrogen to eliminate possible differences in moisture content, there was no consistent difference associated with temperature at which the plants grew except in free amino acid and amide nitrogen contents. This indicates that growth at the lower temperature may have altered the water balance of the plants, so that differences in most of the nitrogenous fractions reported in table I are merely reflections of different moisture contents of the plant tissue. In the case of free amino acid nitrogen and amide nitrogen, however, the increased contents in the hardened plants were so marked that they were apparent when either method of expressing the data was used. These two fractions also were correlated with electrical conductivity ($r = -0.976$ and $-0.981$ for free amino acid nitrogen and amide nitrogen, respectively), which has been used as a measure of cold hardiness of plants (5).

A second experiment was carried out to determine the rate at which cold hardened wheat plants lose their hardiness, and to obtain direct evidence regarding the influence of temperature at which the plants were grown on moisture content. Pawnee wheat was grown for 3 weeks as described above. One portion then was maintained at 70° F, while the other portion was hardened at 35° F for 2 weeks, after which it was returned to the chamber operating at 70° F. Samples were removed for analyses before hardening, after 2 weeks of hardening, and at various intervals during the dehardening phase (fig 1).

After 2 weeks the water content of the plants subjected to hardening was 80%, compared with 85% for the unhardened plants. Thereafter the water

Table I

Changes in Conductivity and Certain Constituents of Winter Wheat During Cold Hardening

| Fraction | Treatment | Weeks | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 0 | 2 | 4 | 6 |
| | | *Mg N/10 g fresh wt* | | | | *% of total N* | | | |
| Total N | U* | 44.3 | 40.6 | 43.7 | 47.5 | ... | ... | ... | ... |
| | H* | ... | 57.8 | 54.0 | 59.6 | ... | ... | ... | ... |
| Soluble N | U | 32.8 | 33.0 | 34.4 | 29.6 | 74.0 | 81.3 | 78.7 | 62.3 |
| | H | ... | 46.6 | 40.6 | 41.7 | ... | 80.6 | 75.2 | 70.0 |
| Soluble protein N | U | 21.2 | 25.0 | 23.2 | 17.3 | 47.9 | 61.6 | 53.1 | 36.4 |
| | H | ... | 30.0 | 27.1 | 26.4 | ... | 51.9 | 50.2 | 44.3 |
| Soluble nonprotein N | U | 11.7 | 8.0 | 11.2 | 12.3 | 26.4 | 19.7 | 25.6 | 25.9 |
| | H | ... | 16.6 | 13.5 | 16.3 | ... | 28.7 | 25.0 | 27.3 |
| Free amino acids | U | 2.9 | 2.9 | 3.1 | 3.3 | 6.5 | 7.1 | 7.1 | 6.9 |
| | H | ... | 8.7 | 7.6 | 7.3 | .. | 15.1 | 14.1 | 12.2 |
| Amide | U | 0.4 | 0.5 | 0.5 | 0.7 | 0.9 | 1.2 | 1.1 | 1.5 |
| | H | ... | 2.5 | 2.0 | 2.3 | ... | 4.3 | 3.7 | 3.9 |
| Ammonia | U | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.5 | 0.5 | 0.4 |
| | H | ... | 0.2 | 0.3 | 0.3 | ... | 0.3 | 0.6 | 0.5 |
| Insoluble N | U | 11.5 | 7.6 | 9.3 | 17.9 | 26.0 | 18.7 | 21.3 | 37.6 |
| | H | ... | 11.2 | 13.4 | 17.9 | ... | 19.4 | 24.8 | 30.0 |
| Conductivity ($ohms^{-1} \times 10^4$) | U | 4.10 | 4.00 | 3.98 | 3.79 | | | | |
| | H | ... | 0.76 | 0.84 | 0.78 | | | | |

*U, unhardened; H, hardened.

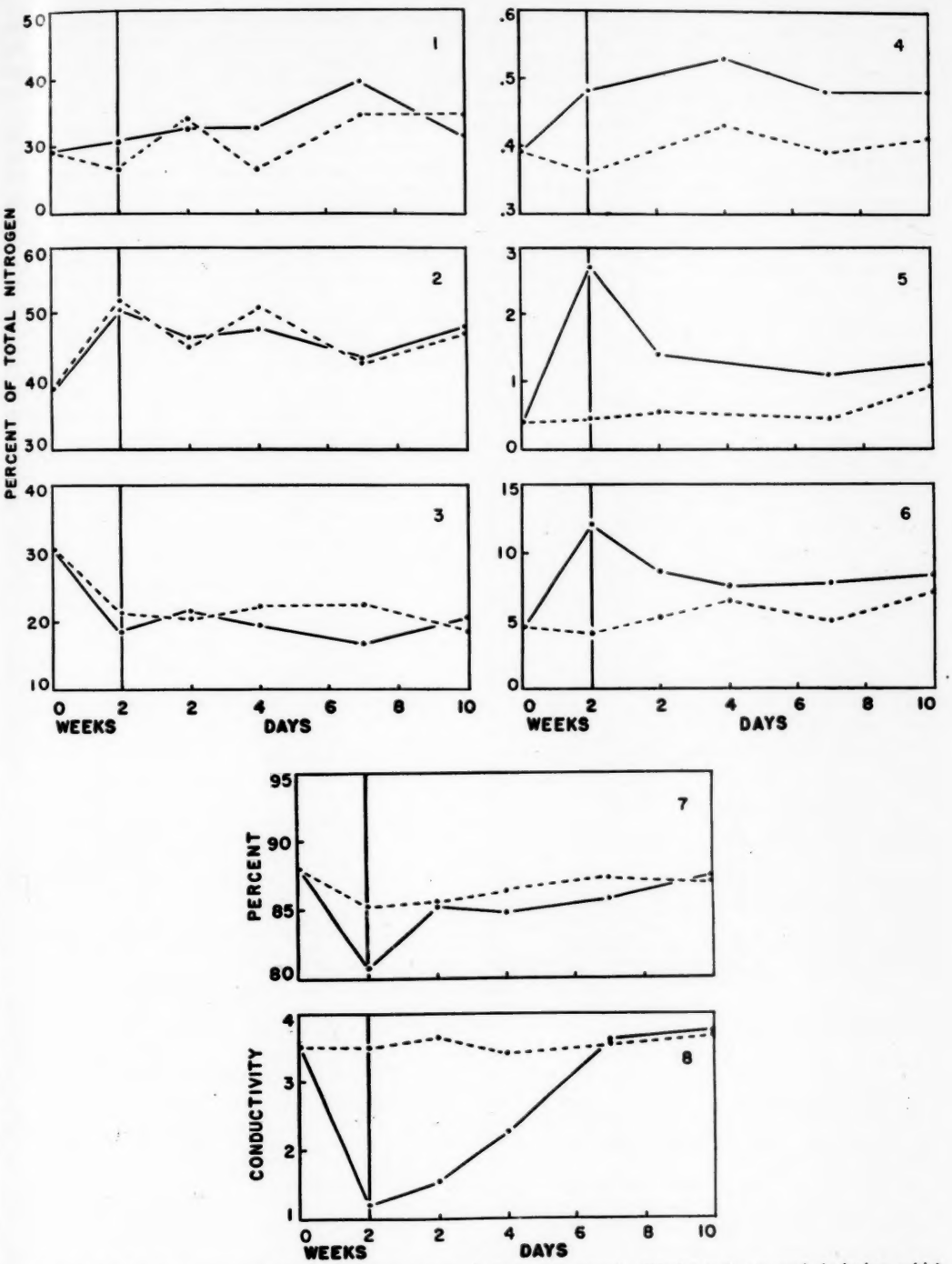



FIG. 1. Solid line represents hardened plants; broken line, unhardened. Weeks indicate period during which one group of plants was hardened; days indicate dehardening period following hardening. 1. Nonsoluble nitrogen. 2. Soluble protein nitrogen. 3. Soluble nonprotein nitrogen. 4. Ammonia nitrogen. 5. Amide nitrogen. 6. Free amino acids. 7. Moisture, (percentages). 8. Conductivity (ohms$^{-1}$ $\times$ 10$^{4}$).

contents of the two lots of plants were essentially identical at a given sampling time. When expressed as percentage of total nitrogen, the various fractions (insoluble N, soluble N, soluble protein N, or soluble nonprotein N) of the two lots of plants were not consistently different at a given sampling date, again indicating that correlations with cold hardiness previously reported were due to moisture differences.

Free amino acids and amides were distinctly higher in the plants hardened for two weeks, again being well correlated with hardiness as measured by conductivity. During the dehardening period these fractions decreased, although they never reached the level of the control plants. During the same period, hardiness, as measured by conductivity, decreased. Sharp decreases in free amino acids and amides, and an increase in conductivity were obtained in two days, indicating that hardiness decreases rapidly when hardy plants are subjected to higher temperatures. Similar results were reported by Laude (9) who found that after 2 weeks of dehardening, field hardened plants could withstand very little more freezing than those grown continuously in the greenhouse.

From the experiments it was not possible to determine definitely the source of the increased amounts of amino acids and amides. However, if a steady state of synthesis and breakdown exists between the protein and amino acid pools at a given temperature, at least two possibilities may be listed: A. An increased rate of proteolytic activity; and B. A decreased rate of protein synthesis from the amino acid pool. Under either condition of amino acid accumulation, amino acid deamination would be expected to result in an increase in amides.

Under the above circumstances, concomitant decreases would be expected in one of the protein fractions. However, the macro-Kjeldahl method of estimating these fractions may not be sufficiently sensitive to show such a decrease consistently. On the other hand, the amino acid and amide methods detect micro quantities of these substances and yield highly reproducible results. The values, therefore, are useful for detecting small changes in nitrogen metabolism. It is recognized that the amino acid method is not specific for amino acids, but may include some peptides (14). However, this does not detract from the usefulness of the method since peptides are intermediates in protein hydrolysis.

## Summary

Wheat plants which were grown in pots in controlled-environment chambers at 70° F were divided into two groups. One group was continued at 70° F, while the other was grown at 35° F.

Soluble protein nitrogen and soluble non-protein nitrogen of the plants grown at 35° F were higher on a fresh weight basis than the corresponding fractions of the unhardened plants grown at 70° F. The water content of the hardened plants was lower than that of the unhardened plants, however, and when these fractions were expressed as percent of total nitrogen, there was no consistent difference between comparable fractions of the two groups of plants. Free amino acids and amides were higher in the hardened plants regardless of the method of expressing the results.

When the cold-grown plants were returned to the warm chamber, their water content increased and free amino acids and amides decreased until both unhardened and dehardened plants had similar contents of these substances after 10 days.

## Literature Cited

1. Association of Official Agricultural Chemists. 1940. Official Methods of Analysis. 5th Ed. Washington, D. C.
2. Beauchene, R. E., A. D. Berneking, W. G. Schrenk, H. L. Mitchell, and R. E. Silker. 1955. The quantitative estimation of amino nitrogen by determination of bound copper with the flame photometer. Jour. Biol. Chem. 214: 731–739.
3. Briggs, D. R., and D. Siminovitch. 1949. Studies on the chemistry of the living bark of the black locust tree in relation to frost. I. Seasonal variations in protein content. Arch. Biochem. 23: 8–17.
4. Bula, R. J., D. Smith, and H. J. Hodgson. 1956. Cold resistance in alfalfa at two diverse latitudes. Agron. Jour. 48: 153–156.
5. Dexter, S. T. 1932. Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol. 7: 63–78.
6. Dexter, S. T. 1935. Growth, organic nitrogen fractions and buffer capacity in relation to hardiness of plants. Plant Physiol. 10: 149–158.
7. Janssen, G. 1929. Effect of date of seeding of winter wheat upon some physiological changes of the plant during the winter season. Agron. Jour. 21: 168–200.
8. Kneen, E. and M. J. Blish. 1941. Carbohydrate metabolism and winter hardiness of wheat. Jour. Agr. Research 62: 1–26.
9. Laude, H. H. 1937. Cold resistance of winter wheat, rye, barley, and oats in transition from dormancy to active growth. Jour. Agr. Research 54: 899–917.
10. Levitt, J. 1956. The hardiness of plants. Amer. Soc. Agron. Monographs, Vol. VI. Academic Press, Inc., New York.
11. Newton, R. 1922. A comparative study of winter wheat varieties with special reference to winter killing. Jour. Agr. Sci. 12: 1–19.
12. Newton, R., and W. R. Brown. 1926. Seasonal changes in the composition of winter wheat plants in relation to frost resistance. Jour. Agr. Sci. 16: 522–538.
13. Pucher, G. W., H. B. Vickery, and C. S. Leavenworth. 1935. Determination of ammonia and amide nitrogen in plant tissue. Ind. Eng. Chem., Anal. Ed. 7: 152–154.
14. Spies, J. R., and D. C. Chambers. 1951. Spectrophotometric analysis of amino acids and peptides with their copper salts. Jour. Biol. Chem. 191: 787–797.
15. Woiwood, A. J. 1949. A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography. Biochem. Jour. 45: 412–417.

## News and Notes

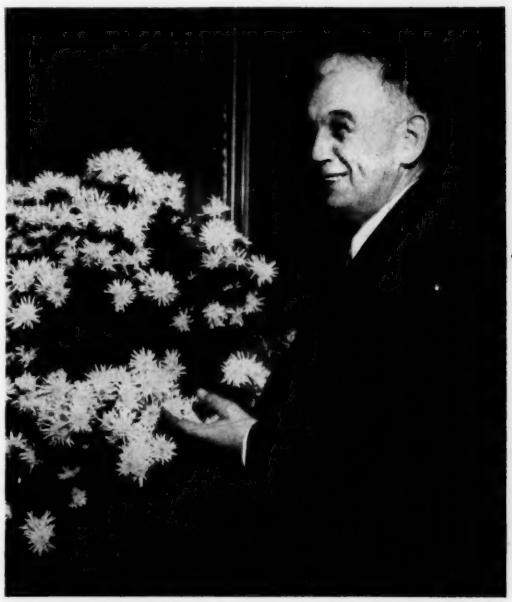
### Ezra Jacob Kraus
1885–1960

When Dr. E. J. Kraus, visiting professor of horticulture, Oregon State College, died February 28, 1960, in Corvallis, Ore., he left behind hundreds of beautiful hybrid chrysanthemums and day-lilies and just as many friends among horticulturists and botanists throughout the country. Next to the late Liberty Hyde Bailey, he was one of the most beloved personalities among plant scientists and experimental horticulturists. He combined, to a high degree, scientific training with its useful application, especially in horticulture. His extensive knowledge of plants, his kindliness and frank honesty and sincerity, attracted to him friends and investigators from many fields of botany, horticulture, and agronomy. He was an inspiration to both graduate and undergraduate students up to the last and helped many financially. Because of his appealing personality, his love of plants, his vast knowledge and non-technical approach to many problems, he had a wide audience among horticulturists. He was popular and at home equally well in scientific circles and garden club meetings. Many students of plant life came under his guidance and obtained their final degrees while Dr. Kraus was a staff member in the Departments of Botany at the University of Wisconsin and the University of Chicago.

Despite his protracted physical disability, that might have justified a complete retirement many years ago, Kraus continued to be self-effacing and always ready to assist others. Few people in technical horticulture were more sought after for friendly advice, criticism and suggestions than Dr. Kraus. And he was never too busy, too absorbed in his own work to place his knowledge and judgment at the disposal of all who felt the need for his help.

Dr. Kraus's primary activity as a teacher and investigator in botany, was in plant morphology, though he became widely known for his and Kraybill's popular physiological research, "Vegetation and Reproduction with Special Reference to the Tomato". This publication won for him wide recognition and acclaim. While the carbohydrate-nitrogen relationship concept, as a basis of plant development, proposed in this publication, is now largely discounted by plant physiologists, as a result of this work and emphasis, attention was focused on the importance and value of fundamental laboratory research with plants. This publication marked the beginning of a scientific approach to many plant cultural problems and marked the establishment of research laboratories in connection with agricultural experiment station activities throughout the country.

During his early horticultural career Kraus was interested in problems of fruit pollination and settings which led to studies of structure and growth of fruits. Morphology apparently remained the primary field of interest throughout his life. In later years, he displayed an active attention to growth regulators, their effects on plants, and their application in agriculture. In fact, he was one of the pioneers in the study of growth regulators, their morphological and histological effects on plants and their potential practical value. This helped greatly to develop 2,4-dichlorophenoxy acetic acid (2,4-D) and subsequently numerous other synthetic chemicals as effective and valuable weed killers and their extensive utilization in crop production. It led to a rapid commercial expansion in the manufacture of special chemicals for weed control throughout the country.



Kraus was born March 19, 1885, in Ingham County, Mich., the son of Christian and Katherine (Baumgrass) Kraus. As with so many other early students of plant life, he obtained the B.S. degree at Michigan State Agricultural College (now Michigan State University) in 1907. After several years of

outstanding service on the faculty of horticulture at the Oregon State College, he obtained, while on sabbatical leave, the Ph.D. degree in Botany from the University of Chicago in 1917. Upon returning to Oregon he was appointed Dean of the Division of Basic Arts and Sciences, but two years later accepted a position as Professor of Applied Botany at the University of Wisconsin, 1919 to 1927. From 1927 until his "retirement" in 1949, he was professor and later chairman of the botany department of the University of Chicago, where he was honored by being officially named Distinguished Service Professor in 1943. Along with his academic work, Kraus held, for many years, (primarily in a consultative capacity) also an appointment as a plant physiologist in the U. S. Dept. of Agriculture. After leaving the University of Chicago in 1949, Dr. Kraus accepted a visiting professorship in horticulture at the Oregon State College in Corvallis where he spent the last ten years breeding and introducing chrysanthemums and other ornamental plants on a large scale, much of the work being done at his own expense.

Dr. Kraus was a member of and gained recognition in many professional societies: American Society of Hort. Science (President 1927); American Society of Plant Physiologists (President 1928); American Association for the Advancement of Science (Vice president and chairman, Sec. G, 1930); American Society of Naturalists (Vice president 1931); Ecological Society of America, and Entomological Society of Washington.

Among other recognitions, he was made an honorary vice president of the American Forestry Association (1947) and was for 12 years on the National Research Council Board of Fellowships. In recognition of his work and service, doctor of science degrees were conferred on him by the Oregon State College in 1938 and by the Michigan State University in 1949. Numerous, indeed, are the national and regional horticultural organizations that have honored Dr. Kraus with medals, awards, and citations of all kinds.

Because of his stern character, humanitarian convictions, and profound honesty, Kraus abstained from all so-called community activities. His attitude toward fellow men was, for lack of a better definition, that of a practicing Christian, of which there seem to be very few remaining specimens. Instead of participating in conventional or popular activities or trumped-up mass emotions, he went his own way, enjoying the beauty of nature, works of art, and human kindness. During the 47 years the writer has known him, he has not come across a more kindly and more unselfish man than Dr. Kraus.—A. E. MURMEEK

NATIONAL SCIENCE FOUNDATION: The Division of Biological and Medical Sciences of the National Science Foundation announces that the next closing date for receipt of basic research proposals in the Life Sciences is September 15, 1960. Proposals received prior to that date will be reviewed at the fall meetings of the Foundation's advisory panels and disposition will be made approximately four months after the closing date. Proposals received after the September 15, 1960, closing date will be reviewed after the spring closing date of January 15, 1961.

Address inquiries to the National Science Foundation, Washington 25, D. C.

CANADIAN SOCIETY: The Canadian Society of Plant Physiologists met in the University of Toronto June 2 and 3, 1960. The proceedings included six sessions of contributed papers and the G. H. Duff Memorial Symposium on Developmental Physiology under the Chairmanship of Dr. G. Krotkov. Participants in the symposium were Dr. E. T. Bünning, University of Tübingen; Dr. F. C. Steward, Cornell University, and Dr. G. Setterfield, National Research Council of Canada. The Society has established a category of corresponding members available to plant physiologists outside Canada. Address inquiries to Dr. Dorothy F. Forward, Secretary-Treasurer, Department of Botany, University of Toronto, Toronto 5, Canada.—DOROTHY F. FORWARD, *Secretary-Treasurer.*

# INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

## I. Writing Manuscript

A. Style In general, the style of an article will reflect the author's preference and habits of writing. The Editor may suggest improvements, but will not insist on rigid conformity to conventions at the expense of an author's individuality. Scientific writing, however, should meet accepted standards of grammar, syntax, and diction.

Strive for specific and concise expression. Use active verbs. Avoid complicated sentence structure. Write in a straightforward manner. Use care in preparing the original manuscript to obviate subsequent delays in publication because of the need for manuscript revision.

B. Length Beyond ten pages (including tables, figures, and literature citations) authors will be billed $20 per page for overrun. Approximately four double spaced typewritten pages (pica type) equal one page of printed type.

C. Footnote References These should be avoided in the body of the text, but if a footnote must be used, it should be numbered with an Arabic numeral and typed at the bottom of the page on which reference to the footnote is made.

D. Acknowledgements Do not include acknowledgements in a footnote; they belong under their own heading following the SUMMARY and preceding LITERATURE CITED.

E. Punctuation

*1. Periods and Commas* These are the most often used and misused punctuation marks; the period is not used enough, the comma too often. Avoid complicated sentences which require many commas, colons, or semicolons. Break long sentences into brief, concise sentences which will be short and easily understood.

*2. Quotation Marks* Avoid the urge to use excessive quotation marks. Often a word is used in a sense slightly different from its ordinary meaning. This does not necessarily warrant the use of quotation marks. In case a term is used in an unconventional way and the ambiguity might lead to misunderstanding, use quotation marks only when the unfamiliar meaning is first explained.

*3. Hyphens* Use sparingly.

F. Subtopics Use this order and style to number subtopics:

I. I)
A. A)
1. 1)
a. a)

G. Scientific Names The complete botanical name (genus, species, and authority for the binomial) of all experimental plants should be included, preferably under MATERIALS AND METHODS. Refer to the most recent International Code of Botanical Nomenclature for rules governing the use of scientific names.

H. Abbreviations In place of certain unwieldy chemical names abbreviations may be used as a convenience. The writing, however, must remain readily comprehensible and unambigious. For the most part only widely used abbreviations should be employed (e.g.: ATP, RNA, TPN). Standard chemical symbols may be used without definition: Ca, NaOH. All others should be defined when they first appear. Example: The reaction was thought to involve flavin adenine dinucleotide (FAD). If your article uses several abbreviated forms it is permissible to define them all in a single footnote where the first abbreviation is introduced.

In no case should the number of nonstandard abbreviations be so large that the reader is forced to learn a special vocabulary in order to read your article easily. Abbreviations should not frustrate the reader; they should be used for his convenience, not the author's convenience.

Avoid abbreviations in title and summary. Because these parts frequently are translated into foreign languages, undefined abbreviations may be confusing.

I. Chemical Formulas It is expensive (sometimes impossible) to set formulas in type. Also there is danger of introducing errors. Submit structural formulas or involved reaction sequences as clearly executed India ink drawings which can be reproduced photographically. Indicate where these are to be inserted into the text. Alternatively a complex reaction diagram or flow sheet may be treated as a regular figure, given a number, and supplied with a legend.

## II. Preparing Typescript

Carefully type copy in accordance with instructions. Give special attention to the form used by the Journal for tables, figure legends, and literature citations. Consult recent editions of PLANT PHYSIOLOGY for proper placement of main headings, paragraph headings, and subheadings, and for the use of abbreviations.

A. Spacing Double space everything, including tables, legends, literature cited, and footnotes. It is essential that all copy be double spaced to allow room for the extensive proofmarking which must

be done (even to a perfect typescript) in the editorial office so the printer will know precisely how to set your copy in type.

Begin typing 1/3 of the way down the first page; leave liberal side margins.

Each table, each set of figures, legends, and literature citations should be doubled spaced on a separate piece of paper; these items are given by the printer to different people for setting in type sizes different from that in the body of the manuscript.

B. ORDER Arrange manuscript copy in this order:

1. Name and address for mailing proofs
2. Title of article
3. Author's name and institution
4. Text
5. Summary
6. Acknowledgements
7. Literature cited
8. Tables
9. Legends for figures
10. Figures

C. PAGING Number all pages consecutively in upper right hand corner. Never use a letter such as 2a, 5D, for paging; such pages are easily lost during processing.

D. PAGE ONE Do not use a title page.

*1. Name and Address* On page 1 above the title of the article, put the name and address of the person to whom editorial correspondence (including galley proof and reprint order form) is to be sent. The name and address of the institution where the work was done follow the name of the author directly below the title.

*2. Footnotes* One or more footnotes are included at the bottom of the first page. The first footnote (indicated by superscript 1 after the title) gives the date the manuscript was received. The second footnote credits the source of financial support (if any). Additional footnotes give the present address of the author, if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used.

E. NUMBERS

1. Write out all numbers, cardinal and ordinal, from one through ten. Use figures for all numbers larger than ten. Exceptions:

*a.* When using precise measurements such as 5 per cent, 6 mm, 8-hour cycle, figure 1. In nonprecise usages spell numbers out: about two weeks, six or eight degrees.

*b.* In a series of related numerical expressions treat all alike even though violating the general rule: 2 leaves, 8 leaves, and 12 leaves.

*c.* When a single expression has consecutive numbers, use both figures and spelled out numerals: two 6-min exposures.

*d.* Spell out ALL numbers or fractions which begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral.

*e.* Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, three to ten degrees. Exceptions: tables, figures, graphs.

F. FRACTIONS Write out and hyphenate nonprecise simple fractions: two-thirds, *but* half strength. Exceptions: tables, figures, graphs, legends, fractions in parentheses. Often it is desirable to use decimals instead of fractions. For values less than one, insert a zero before the decimal point: 0.7 g *not* .7 g.

G. LITERATURE CITED This section of a manuscript requires extensive proofmarking even though copy is correct in every detail. Typing errors are easily made, but difficult to detect, so exercise special care in preparing and checking the bibliography. Since they are set in a typeform different from that used for the body of the manuscript, literature citations should be *double spaced on a separate piece of paper*. Consult the most recent issue of PLANT PHYSIOLOGY for Journal style. Note the limited use of capitals.

Check all citations (such as title, volume number, pages, and date) for completeness. Check foreign titles for diaresis and other markings. Make certain that all citations are referred to in the text.

List citations in alphabetical order (not in order of reference), then number them and indicate citations in the body of the text by Arabic numerals enclosed in parenthesis.

Unpublished data, personal communications, and articles in preparation are not acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press may be so designated in LITERATURE CITED. An article is not properly referred to as "in press" unless it has been *accepted* for publication. The journal in which an in press article will appear should be included in the literature citation.

*1. Authorship* List family name of first or sole author before listing initials or given name. Thereafter write names and initials (or given name) of all coauthors in their natural order. Use initials for a man's given name; write out a woman's given name: Reeves, A.C., R. D. Jones, and Helen Smith.

*2. Date* The year belongs immediately after the author's name.

H. HYPHENS Avoid dividing a word at the end of a line. Such division increases the likelihood of a printer's error.

I. UNDERLINING In general, do not underscore anything. When copy is marked for the printer,

headings, scientific names, etc., will be underscored in the Editor's office. Exception: *rarely* a word or phrase may be underlined for appropriate emphasis.

J. SPELLING Draw a box around unusual spelling to indicate that the word has been spelled correctly.

## III. PREPARING TABLES

Authors should set up their tables in a form consistent with recent issues of PLANT PHYSIOLOGY. Number tables consecutively in Roman numerals and place the author's name on the back of each page.

A. COPYFITTING Tables should be preplanned to fit one or two printed columns (3 inches or 6⅛ inches wide, respectively) and not to exceed 8½ inches in height, including heading, body, and footnotes. Tables may not be rotated 90°.

On the average, about 52 typewriter spaces (excluding the new executive style of typewriter) fill one 3-inch line of type of the size normally used in tables; ten double spaced typewritten lines require 1 inch of the available vertical printed space.

B. NUMERALS Check tabular data as well as numerical values reported in the text for the proper number of significant figures. Frequently authors report more digits than are significant. Use three dots (*not* a dash) to indicate a blank in a table: ... For decimals smaller than one, insert a zero before the decimal point: 0.349.

C. FOOTNOTE DESIGNATIONS Refer to footnotes by the asterisk, double asterisk, triple asterisk, dagger, double dagger, and section, in this sequence, as needed: *, **, ***, †, ††, ‡. *Place all explanatory material in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

D. HEADINGS AND FOOTNOTES Wherever possible, omit unnecessary articles such as "the", "a", in headings and notes. Also avoid bulky prepositional phrases.

## IV. ILLUSTRATIONS

A. PRINTS FOR REVIEW For manuscript review it is satisfactory to furnish illustrations which have been reproduced by such economical copy methods as Verifax and blue print. Thus prints which will be used for publication need not be sent through the mail repeatedly. *Do not send original drawings or photos* at any time.

B. PRINTS FOR REPRODUCTION After your manuscript has been accepted for publication you must submit good quality glossy prints (glossies) for making electrotypes.

*1. Kinds of Illustrations* Figures are of two kinds, line drawings and halftones. Line drawings are just that—line drawings usually done with India ink on white paper. Halftones, in contrast to line drawings, are not just black and white, but they also are shaded from black to white. Because halftones are photographed through a screen some detail is lost in reproduction.

*2. Special Treatment of Photographs* For illustrations in which it is especially important to bring out all the detail present in the original photograph special processing will be undertaken. Should your paper contain electron-micrographs, radioautographs, or other material whose reproduction should be deemed critical, call the editor's attention to the need for special processing.

*3. Good Illustrations* Plan in advance for clear, legible illustrations. Line drawings should be intensely black on white; halftones should have good contrast and be in sharp focus throughout. No glossy should contain blemishes. Do not use paper clips on photos; grooves, gouges, and creases show when reproduced.

Generally the appearance of a graph is improved if the top and right sides are boxed in by lines of the same weight as the abscissa and ordinate.

Guided lettering is preferable to hand lettering or typing.

*4. Size* Although the Editor determines the final printed size of an illustration, an author should plan for maximum reduction. If symbols, such as triangles, circles, and squares (or letters and numerals) are not sufficiently large to begin with, they may become mere blurs when reproduced. Any letter smaller than 1 mm may be illegible. Exceedingly large symbols, thick lines, or heavy numerals may become unsightly when reduced.

Figures are reproduced the same size (x 1) or they are reduced. (Enlarging magnifies defects.) Illustrations are fitted either into 3 inch or 6⅛ inch column widths. Although occasionally an illustration must be 8½ inches high, this is undesirable. One should allow space at the bottom for the figure legend. It may help you to sketch a rough onion skin overlay of a typical Journal page layout for estimating final reduced size of an illustration. Final size, however, cannot be estimated until after illustrations are properly grouped and mounted.

*5. Grouping* One large illustration is cheaper to reproduce than three to six small ones, so *as far as possible group all figures.* Mount similar figures close together. Place halftone and line drawings in separate groups.

Limit the area of each photograph to that which is necessary to show pertinent details. Trim off irrelevant portions and minimize blank space.

*6. Mounting* Mount all illustrations with rubber cement on white cardboard or stiff white paper. Total size should not exceed 9 x 11

inches. Anything larger than an ordinary file folder is easily mutilated; it also requires special packaging and handling.

7. *Identification* Place the author's name on the back of each figure and legend. Place the figure number on the back of each figure. Identification insures against misplacement.

## V. Submitting Manuscripts

Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

## VI. Processing Manuscript

A. Review The manuscript will be sent usually to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

B. Revision Manuscripts returned to the author for revision should be sent back to the Editor within two months; otherwise the paper will be published under the date of receipt of the revised manuscript and not the original date.

Retype each page on which there are extensive changes.

C. Galley Proof This will be sent to the person designated on page 1 above the title. (See II D 1).

1. *Returning Proofs* Return only one set of galley proofs to Editor. Keep any other set for reference. Be certain to notify Editor promptly of change of address, so galleys can be sent to the correct address.

2. *Charges for Errors* You will not be charged for correcting printer's errors, BUT you will be charged at cost for all changes or corrections in galley which are your fault. Here are two ways to minimize charges:

*a. Proofread Manuscript* Check ALL references carefully BEFORE submitting manuscript to Editor. Corrections in typing are easy to make, whereas corrections in galley or page proof are expensive and sometimes difficult to make.

*b. Corrections* Where a correction is optional remember that a change made near the beginning of a paragraph can necessitate resetting every subsequent line to the end of the paragraph. The printer charges for each line he must reset to correct an error, so weigh the cost against the importance of the change you propose.

D. Delays In general, a manuscript should be reviewed and returned to you with the Editor's decision within two months. After you send a revised manuscript to the Editor allow 2½ months for galleys to be sent you for correcting. Should you find that processing your manuscript has taken an unusually long time at either stage, it would be well to inquire of the Editor as to the cause of the delay. (This is a precaution against unrecognized loss of manuscripts in the mail—a rare event, but it happens.)

E. Errata If necessary, an errata will be published for each volume. Authors therefore are requested to call the attention of the Editor to any significant errors in their published manuscripts.